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14. ABSTRACT-Characteristics of aggressive prostate cancers (CaP) include a loss of sensitivity to physiologic levels of TGF-β due to in part TGF-β receptors (TβRs) methylation mediated by DNA methyltransferase (DNMT). However, the mechanisms underlying these alterations remain undetermined. We used human CaP cell lines with varying degrees of invasive capability, and human CaP samples to elucidate the mechanism(s) associated with TGF-β insensitivity and disease outcome following radical prostatectomy. We determined that more aggressive CaP cells had significantly higher TGF-β levels and increased expression of outcome following radical prostatectomy. We determined that more aggressive CaP cells had significantly higher TGF-β levels and increased expression of cells. Blockade of TGF-β signaling or the extracellular signal-regulated kinases (ERK) was associated with a dramatic decrease in the expression of DNMTs, and with a coincident increase in the expression of TβRs in cancer cells. In addition, there was a time dependent positive correlation between treatment of cells with TGF-β and the expression of p-ERK in CaP cells. In contrast, benign prostate cells demonstrated a negative correlation between TGF-β treatment and p-ERK expression. Inhibition of TGF-β in an in vivo xenograft model using 1D11 was associated with inhibition of tumor growth but also the downregulation of p-ERK and DNMTs. Finally, independent of Gleason grade, TGF-β induced expression of DNMT1 was associated with biochemical recurrence following radical prostatectomy. Our findings demonstrated that CaP tumor derived TGF-β may induce the expression of DNMTs which subsequently results in the hypermethylation of its own receptors and insensitivity to growth inhibition. ERK activation mediates this feedback loop which is associated with the aggressive potential of CaP. In addition, this pathway may have future clinical implications in CaP as a therapeutic target and a prognostic tool.

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INTRODUCTION:

TGF-beta is a family of pleiotropic growth factors with diverse functions (Massague, 1990). The biological effect of TGF-beta is mediated through TGF-beta receptors and down stream transcription factors, Smad molecules (Massague et al, 1992; Derynck and Feng, 1997). It has been well know for at least 10 years that TGF-beta is a tumor suppressor in benign cells and at the early stage of carcinogenesis but it facilitates tumor invasion and metastasis in advanced tumors (Barrack 1997; Wang 2001; Li et al, 2005; Jakowlew, 2006; Pardali and Moustakas, 2007). However, the exact mechanism of this paradoxical action of TGF-beta between benign and malignant cells remains incomplete. In the present proposal, we will address this paradigm with a new perspective from the point of view of the status of ERK activation upon TGF-beta action.

The use of TGF-beta antibody for treating cancers has been reported in the past with mixed results (Border et al, 1990; Shah et al, 1995a, b; McCormick et al, 1999). However, since the use of TGF-beta antibody for treating cancer is now underway in a FDA approved clinical trial, many safety and efficacy concerns associated with the use of this antibody must be resolved. (See NIH Controlled Trials Web site page: hase I study of the safety and efficacy of GC1008: A human anti TGF Beta monoclonal antibody in patients with advanced renal cell carcinoma or malignant melanoma) Therefore, there is a heightened and urgent need to re-investigate the use of TGF-beta antibody for treating cancers, especially, when our knowledge on TGF-beta action has been increased through the years (Biswas et al, 2007). In addition, TGF-beta antibody can not only be used as a therapeutic tool for treating cancers; but can also be used to elucidate the mechanisms of action of TGF-beta in tumor progression and metastasis. Our recent discovery of the status of ERK activation in TGF-beta action is exciting and the current proposal will address the most critical aspect regarding the mechanism of tumor evasion of the immune surveillance and of down-regulation of TGF-beta receptors, both of which will lead to tumor progression.

TGF-beta antibody can be considered as a double edged sword, when used for treating cancers. If tumors are sensitive to TGF-beta, there is a possibility that a sub-optimal depletion of TGF-beta within the tumor microenvironment may facilitate tumor progression. Our animal studies showed that a high dose of TGF-beta antibody was necessary to control tumor growth (Liu et al, 2007; Perry et al, 2008). However, at the same time, a high dose of TGF-beta antibody may pose a safety risk to the recipient. In view of recent approval by the FDA for Phase I clinical trials for cancer patients, the trial called for the use of the lowest possible dose for cancer patients. In any clinical trial, the safety and efficacy of using TGF-beta antibody for treating cancer must be carefully evaluated. Although the literature has not recorded any noticeable side effects when TGF-beta antibody is used (Ruzek et al, 2003; Prud'homme, 2007), possible end organ hyperplasia (such as in the prostate and the stomach) has not been ruled out (Bhowmick et al, 2004).

In general, TGF-beta is growth suppressive. A characteristic feature of cancer cells is a loss of sensitivity to TGF-beta, resulting in a loss of growth suppression (Diaz-Chavez et al, 2008). The loss of sensitivity to TGF-beta in cancer cells is mainly due to down-regulation of TGF-beta receptors. Prostate cancer is no exception. We found more than 10 years ago that there is a universal down-regulation in TGF-beta receptors in prostate cancer (Kim et al, 1996) and the degree of this down-regulation is correlated with disease progression and clinical outcome (Kim et al, 1998). This observation was confirmed by others (Cardillo et al, 2000; Zeng et al, 2004). However, the mechanism of this down-regulation of expression of TGF-beta receptors in prostate cancer remains to be elucidated. Recently, we have observed that down-regulation in TGF-beta receptors in prostate cancer is mainly due to promoter hypermethylation (Zhang et al, 2005). Further, results of our recent study suggested that TGF-beta induced ERK activation could be a possible mechanism for down-regulation of TGF-beta receptors in prostate cancer through promoter hyper-methylation (see below).

Promoter hypermethylation of a host of genes has been reported in prostate cancer patients and has been linked to poor prognosis (Rosenbaum et al, 2005). However, the mechanism of such wide spread promoter hypermethylation in cancer remains unclear. ERK can be activated by TGF-beta in cancer cells (Ellenrieder et al, 2001; Huo et al, 2007). Since activated ERK is a potent inducer for DNA methylatransferases (Oelke and Richardson, 2004; Lu Q et al, 2005; Lu R et al, 2007), we speculate that TGF-beta signaling can modulate the status of promoter methylation of many key genes in cancer progression, including TGF-beta receptors. Results of our preliminary study revealed that TGF-beta can differentially activate ERK in benign and cancerous epithelial cells. In our preliminary study, we found that, in prostate cancer cells, ERK can be activated through TGF-beta signaling.

The above discussion has allowed us to propose the following hypothesis to address the mechanism of down-regulation of TGF-beta receptors in prostate cancer cells. We hypothesize that prostate cancer cells, in response to TGF-beta stimulation, will down-regulate the expression of TGF-beta receptors through ERK activation, which in term induces the expression of DNA methyltransferases leading to promoter hyper-methylation of the TGF-beta receptor genes and down-regulation of expression of TGF-beta receptors. Since epigenetic events can be reversed, we will determine if the use of TGF-beta antibody can reverse this down-regulation of TGF-beta receptors in prostate cancer cells. Many advanced tumors are immunosuppressive due, at least in part, to their ability to secrete large amounts of TGF-beta (Letterio and Roberts, 1998; Wojtowicz-Praga, 1997, 2003; Pinkas and Teicher, 2006). Since TGF-beta is a potent immunosuppressant, a gradient of TGF-beta surrounding the tumor site renders the host immune surveillance system ineffective against tumor cells (de Visser and Kast, 1999). However, despite intense investigation on this topic, the mechanism of TGF-beta in immune suppression remains unclear. The recent recognition that CD4+CD25+ T regulatory cells can suppress anti-tumor immunity has offered a mechanism for tumor evasion of host immune surveillance (Sakaguchi et al, 1995; Shimizu et al, 1999; Sasada et al, 2003). We have demonstrated the generation of CD4+CD25+ T regulatory cells in the tumor parenchyma through tumor-derived TGF-beta (Liu et al, 2007). Others have confirmed our results in different tumor systems (Jarnicki et al, 2007; Selvaraj and Geiger, 2007). This discovery is exciting as it explains at least in part the role of TGF-beta in evasion of host immune surveillance program by the tumor cells. Most recently, we have further discovered that Foxp3 expression (a marker for T regulatory cells) is mediated through TGF-beta induced ERK inactivation (Luo X et al, 2008). These discoveries describe a new paradigm in TGF-beta action in evasion of the host immune surveillance by tumor cells; and, in the present proposal, we propose to test if ERK inactivation actually mediates promoter de-methylation in the Foxp3 gene.

Our recent discoveries indicated that tumor derived TGF-beta was responsible for the generation of CD4+CD25+ T regulatory cells from CD4+CD25- T cells (Liu et al, 2007) and that TGF-beta inactivates ERK in T cells which led to the expression of Foxp3, a marker for T regulatory cells (Luo X et al, 2008). These findings are exciting and allow us to postulate the hypothesis that tumor-derived TGF-beta leads to ERK inactivation and to promoter de-methylation in the Foxp3 gene of T cells. The use of TGF-beta antibody will reverse this process. This hypothesis is proposed with the understanding that activated ERK is a potent inducer of DNA methyltransferases (Oelke and Richardson, 2004; Lu Q et al, 2005; Lu R et al, 2007). Using neutralizing TGF-beta antibody for treating cancer is a plausible approach, as it alters the tumor micro-environment. In light of the current on-going Phase I clinical trial, the safety and efficacy of using TGF-beta antibody must be carefully evaluated in a pre-clinical setting. The use of TGF-beta antibody may actually pose a risk of promoting certain cancers, when the particular cancer is sensitive to TGF-beta and low doses are administered. Using TGF-beta antibody can also allow us to elucidate mechanisms of TGF-beta action. The status of TGF-beta mediated ERK activation in tumor cells as well as in T cells plays a critical role in tumor progression and metastasis. A successful conclusion of this research will not only allow us to gain new insights into the role of TGF-beta mediated ERK activation in prostate cancer progression but also will pave the way for a clinical trial in prostate cancer patients using the humanized TGF-beta antibody (GC1008; Genzyme Corporation).

At the time of this report, we have completed studies described in Aim 1, Aim 2 and most of Aim 3. Currently, studies described in Aim 3 are underway. Briefly, the progress can be summarized by our paper published recently (Zhang et al, 2011). In the past year, we reported that anti-Transforming growth factor-Beta antibody 1D11 suppresses the invasion of human prostate cancer cells. Furthermore, the differential response to TGF-beta in malignant cells will lead to an up-regulation of DNMT, which will result in a down-regulation of TGF-beta type II receptor in malignant cells. Final, the recruitment of PP2A by TGF-β receptors mediates the response to TGF-β-induced activation of ERK in prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS (2011-2012):

Our new data for Specific Aim 3 demonstrated that neutralizing TGF-beta antibody (1D11) can prevent human prostate cancer metastasis, mainly through the prevention of Erk activation, which subsequently presented DNA hypermethylation in the target cells. TGF-β induced, r mediated expression of DNMTs is associated with biochemical recurrence in prostate cancer patients after radical prostatectomy. The recognition of TGF-beta mediated DNA hypermethylation is indeed exciting and novel. Meanwhile, we found the recruitment of PP2A by TGF-β receptors mediates the response to T -β-induced activation of ERK in prostate cancer. (Zhang Q et al. 2011 AACR). We will devote the remainder of the funding period to investigate the mechanism of regulation of DNMT expression by Erk activation in response to TGF-beta in cancer cells.

BODY OF REPORT (2011-2012):

We have completed all the approved SOW In Specific Aim 1 and Specific Aim 2, and submitted the progress reports. The tasks accomplished in Year 3 are focused on Specific Aim 3.

Specific Aim 3: Up-regulation of TGF-beta receptors by TGF-beta antibody in prostate cancer cells.

We discovered that, in cancer cells, the status of ERK activation dictates their response to TGF-beta. Again, it known that tumor cells have a reduced sensitivity to TGF-beta, which is associated with an aggressive phenotype (Kim et al, 1996, 1998). Although we knew that promoter hypermethylation was the cause of this event (Zhang et al, 2005a), the reason for this promoter methylation is unclear. Recently, we found that treatment of cancer cells, not benign cells, with TGF-beta resulted in activation of ERK. This discovery allows us to propose the hypothesis that cancer cells, in response to TGF-beta, will activate ERK, which will down-regulate the expression of TGF-beta receptors through promoter methylation and that treatment with TGF-beta antibody can reverse this process. The summary of progress listed as following:

* The most significant finding of the current study has been that tumor derived TGF-β could induce the expression of DNMTs which subsequently results in the hyper-methylation of its own receptors and insensitivity to growth inhibition. Treating these cancer cells with TGF-beta antibody could reverse TGF-beta receptor expression.

* In light of a recent publication in Nature Genetics (Hansen et al, 2011), our recognition of TGF-beta mediated DNA hypermethylation has created an opportunity for additional investigation. Our study (2011-2012) focused on mechanisms of regulation of DNMT expression by Erk activation in response to TGF-beta in cancer cells. Here we report the new findings (2011-2012) in this Specific aims.

<u>Anti-Transforming growth factor-Beta antibody 1D11 suppresses the invasion of human prostate cancer cells. (Zhang Q et al. 2011 AUA)</u>

Metastases are responsible for disease specific mortality in men with prostate cancer (CaP). Previously, we reported that TGF- β induced vimentin expression is associated with the epithelial-to-mesenchymal transition (EMT) which is correlated with migration of CaP cells and a worse prognosis in clinical specimens obtained at prostatectomy. In this study, we explored whether a specific neutralizing anti-TGF- β antibody 1D11 inhibits the migratory and invasive potential of CaP cells. In this study, cell invasion assays were performed using the human CaP cell lines DU145 and PC-3 in a 24-well matrigel transwell chamber (8-µm pore size; CytoSelectTM; Cell Biolabs). Four different treatment groups were assigned to each cell line: Group 1- no treatment (control); Group 2- treatment with TGF- β (10ng/mL) for 24 hours; Group 3- treatment with anti-TGF- β (1, -2, -3) neutralizing mAb clone 1D11 (5ug/mL) for 24 hours; and Group 4- treatment with ERK inhibitor U0126 5µM. After 24 hours of invasion, the invaded cells were counted with a light microscope under high magnification objective (x100) and were then lysed and measured at OD 560nm in a plate reader after treatment with the extraction solution. Furthermore, Western blot analyses were performed to evaluate the expression of vimentin in each treatment group. We found an average of 82 cells/field were found to invade in the control group. In Group 2, there was a significant increase in the number of invaded cells (139.33/field) with TGF- β treatment.Invasion of PC3 cells was significantly inhibited by treatment of 1D11, (only 7.67 cells/field). The ERK inhibitor, U0126, was also associated with a significant decrease in the number of invasive cells (14.67 cells/field; Group 4). Consistent with the results of the migration assay, Western blot analyses showed that after treatment with TGF-β, the expression of vimentin increased by 2-2.5 folds. In contrast, with the treatment of 1D11 and U0126, the expression of vimentin was suppressed 70% and 40% respectively. Furthermore, treatment of 1D11 resulted in the 50% inhibition of p-ERK, which indicated that 1D11

may inhibit TGF- β induced vimentin through a p-ERK pathway. The present findings indicate that neutralization of tumor secreted TGF- β by 1D11 may inhibit TGF-β and ERK induced expression of vimentin. Phenotypically this results in the suppression of CaP migration. Our results suggest potential targets for the future development of effective anti-tumor therapeutic strategies.

DNMTs expression is mediated through a phosphorylated-ERK dependent pathway. (Zhang Q, et al 2011, PloS. One). In this study, as we found first, the benign BPH-1 and RPWE-1 cells express significantly higher baseline levels of p-ERK than the PC-3 cells. There is a time dependent positive correlation between treatment with TGF-β1 and the expression of p-ERK in PC-3 cells. The levels of p-ERK continue to increase during all subsequent time points up to 30 minutes after TGF-β1 addition. In contrast, the expression of p-ERK is rapidly (<5 minutes) inhibited after TGF-β1 exposure in benign cells in a fashion that is independent of the total R protein expression. Second, Immuno-fluorescence reveals that only cells (this is PC3 for example) expressing p-ERK exhibit DNMT expression. In contrast, when PC-3 cells are rendered insensitive to TGF- 1 by TβRIIDN, levels of both p-ERK and DNMT are significantly reduced (magnification: 10×20). Third, we performed real time PCR to better quantify the relationship between TGF-β1, p-ERK and DNMTs. Exposure to TGF-β1 significantly increased the expression of all three DNMTs in PC-3 cells. Treatment with 1D11, or MEK inhibitor, UO126 is associated with the down-regulation of all DNMT mRNA expression. Final, there was a significant increase in cell motility through a Matrigel-coated polycarbonate membrane under the treatment of TGF-β1 (1 ng/mL). The invasion of all CaP cells could be inhibited by blocking the TGF-β signal by 1D11 or using a p-ERK inhibitor UO126, or DNMT inhibitor 5-Aza separately. The inhibition of invasion by UO126 can't be reverted by TGF-β treatment. pper right panel orresponding numbers of invasive cells. ottom right panel absorbance values. This result indicates p-ERK mediated TGF-β-induced DNMT potentiates the invasive ability of prostate cancer cell lines.

In vivo validation of the effects of TGF-β on R activation, DNMT expression, and prostate cancer growth.

To validate whether TGF-β is responsible for the activation of R and up-regulation of DNMTs which may be involved in tumor progression in vivo, we conducted experiments using a mouse xenograft CaP model which involved the injection of CaP tumor cells (TRAMP-C2 cells stably transfected with a HSV1-tk-GFP-luciferase reporter, 5×10^6/each mouse). Tumor growth was followed using luciferase imaging. We used three groups of mice to better understand the effects of TGF-β on R activation and DNMT expression roup 1 mice (n = 10) received regular injections of the TGF-β neutrali ing antibody, 1D11. roup mice (n = 10) received the isotype control antibody, 13C4, at the same regular intervals as Group 1. Group 3: received no treatment after xenograft injection as a control. We found that tumor growth was significantly inhibited with anti-TGF-β 1D11 antibody, treatment (Group 1) compared with the other two groups. In fact, at the end of the 45-day treatment period, one of the ten mice (10%) in this group was free of tumor. In the remaining 9 mice, the average tumor weight and volume was 5.3 g and 6.85 cm^3, respectively. In comparison, tumors were found in all mice in Groups 2 and 3. The average weight and volume of tumors in the 10 animals treated with the control antibody (Group 2) or no treatment (Group 3) was significantly greater. There were no metastases in all the groups as assessed by bioluminescence imaging. Immunohistochemical analyses of the primary tumors revealed that the expression of p-ERK and DNMTs in animals in Group 1 were significantly lower than those of the other two groups.

The recruitment of PP2A by TGF-β receptors mediates the response to T -β-induced activation of ERK in prostate cancer. (Zhang Q et al. 2011 AACR)

More recently demonstrated that TGF-β mediated phosphorylation of extra-cellular signal-regulated kinase (ERK) activation results in the prostate cancer (CaP) progression and metastasis. Serine/threonine protein phosphatases 2, PP2A (including subunit -A, -B and -C) are well known to be involved in the dephosphorylation and inactivation of ERK. In this study, we determined the association between the recruitment of PP2A by TGF-β receptors (TβRI and TβRII) and activation of RK under the treatment of TGF-β. In this study, the human CaP cell lines PC-3 with different capability of aggressive (PC-3, PC-3M, PC-3M-Pro and PC-3M-LN4), and benign prostate epithelial cell line BPH-1 were used for these studies. Cells were treated with TGF-β (1 ng m) for minutes. The expression of phospho-ERK (p-ERK), total-ERK (t-ERK), TβRs was evaluated by uantitative western blot analyses. The conjugation of PP2A (-A, -B and -) and TβRI and TβRII were elucidated using western blot following immunoprecipitation (I , ierce rosslin it) with TβRI and TβRII as the precipitant respectively. riefly, precleared lysate was immunopreciptated by the crosslinked TβRI or TβRII antibody (g) and agarose mixture for overnight on . ontrol agarose resin in the it was used as a negative control when western-blot for was conducted. The recruitment of by TβRs was correlated with the expression of p- R and TβRs. We found TGF-ß treatment resulted in an increase in p-Erk

expression (4-fold) in all PC-3 cell lines in a time dependent manner post TGF-ß exposure. In addition, the expressions of TßRI and TßRII were suppressed by 46% and 29% respectively. IP studies revealed recruitment of PP2A conjugated with TßRI and TßRII. In contrast, the expression of p-Erk was dramatically inhibited in BPH-1 by TGF-ß exposure. Although there is no significant change on the expression of TßRs, the ratio of PP2A versus TßRII was significantly increased from 2.08 to 3.12, which suggests that the recruitment of PP2A was relatively increased in BPH-1 cells under the treatment of TGF-ß. Finally, there was a reverse correlation between recruited PP2A and activation of p-ERK in both PC-3 cell lines, and BPH-1 cells.

Disscusion: In summary, our findings indicate that DNMTs expression levels are correlated with invasive capabilities in cultured human CaP cell lines. Additionally, we found that tumor-derived TGF-β and ERK are involved in the regulation of DNMTs in these cell lines. Inhibition of TGF-β in vivo results in the corresponding inhibition of DNMTs, and appears to significantly decrease tumor growth. In addition, we confirmed that the expression levels of TGF-β, R and DNMTs in tissue specimens obtained at the time of prostatectomy mimicked our findings in cell culture. Furthermore, our results suggest that TGF-β suppresses the recruitment of PP2A by TGF-β receptors in a cells in contrast to benign prostate cells, which results in relatively increased activation of ERK and the subsequent tumor progression. The identification of the recruitment of by TβRs represents a new focus to elucidate in part how T -β plays a different, or even a contrary role in CaP and benign cell respectively. PP2A may be a potential new target for CaP therapies. Neutralization of tumor secreted TGF- β by 1D11 may inhibit TGF-β and ERK induced expression of vimentin. Phenotypically this results in the suppression of CaP migration. Our results suggest potential targets for the future development of effective anti-tumor therapeutic strategies.

REPORTABLE OUTCOMES:

As a result of this research funded by the Department of Defense, we have completed two manuscripts (see appendix).

CONCLUSIONS: Our findings indicate that tumor cell-derived TGF-beta may induce the insensitivity to growth inhibition by this cytokine. There is a feedback loop appears to regulate the expression of TGF-beta Receptors through both ERK activation and DNMTs expression and appears to impact on the invasive potential of prostate cancer cells. In addition, this pathway appears to have clinical utility as both a therapeutic option and a prognostic tool. Inhibition of TGF-beta in vivo appears to significantly decrease tumor growth and the number of cancer cells which express either TGF-β, p-ERK, and DNMT. Finally, high levels of TGF-beta-induced expression of DNMT1 may potentially be used to reliably predict biochemical recurrence in patients following radical prostatectomy. TGF-β suppresses the recruitment of by T -β receptors in a cells in contrast to benign prostate cells, which results in relatively increased activation of ERK and the subsequent tumor progression. The identification of the recruitment of by TβRs represents a new focus to elucidate in part how TGF-β plays a different, or even a contrary role in a and benign cell respectively. may be a potential new target for CaP therapies.

REFERENCES:

Barrack ER. (1997) TGF-beta in prostate cancer: A growth inhibitor that can enhance tumorigenicity. Prostate 31:61-70.

Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL. (2004) TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303:848-851

Biswas S, Guix M, Rinehart C, Dugger TC, Chytil A, Moses HL, Freeman ML, Arteaga CL. (2007) Inhibition of TGF-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. J Clin Invest 117:1305-1313.

Border WA, Okuda S, Languino LR, Sporn MB, Ruoslati E. (1990) Suppression of experimental glomerulonephritis by antiserum against transforming growth factor b1. Nature 346:371-374.

Cardillo MR, Petrangeli E, Perracchio L, Salvatori L, Ravenna L, Di Silverio F. (2000) Transforming growth factor-beta expression in prostate neoplasia. Anal Quant Cytol Histol 22:1-10.

Dernyck R, Feng X (1997) TGF-b receptor signaling. Biochem et Biophs Acta 333:F105-F150.

de Visser KE, Kast MW. (1999) Effects of TGF-beta on the immune system: implications for cancer immunotherapy. Leukimia 13:1188-1199.
Diaz-Chavez J, Hernandez-Pando R, Lambert PF, Gariglio P. (2008) Down-regulation of transforming growth factor-beta type II receptor (TGF-betaRII) protein and mRNA expression in cervical cancer. Mol Cancer. 2008 Jan 9;7:3.
Ellenrieder V, Hendler SF, Boeck W, Seufferlein T, Menke A, Ruhland C, Adler G, Gress TM. (2001) Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res 61:4222-4228.
Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP. Increased methylation variation in epigenetic domains across cancer types. Nat Genet. 2011 Jun 26;43(8):768-75.
Huo YY, Hu YC, He XR, Wang Y, Song BQ, Zhou PK, Zhu MX, Li G, Wu DC. (2007) Activation of extracellular signal-regulated kinase by TGF-beta1 via TbetaRII and Smad7 dependent mechanisms in human bronchial epithelial BEP2D cells. Cell Biol Toxicol 23:113-128.
Jakowlew SB.(2006) Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev 25:435-457.
Jarnicki AG, Lysaght J, Todryk S, Mills KH. (2006) Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. J Immunol 177:896-904
Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Sensibar JA, Kim J H, Kato M, Lee C. (1996a) Genetic change in transforming growth factor-beta (TGF-b) receptor type I gene correlates with insensitivity to TGF-b1 in human prostate cancer cells. Cancer Research 56:44-48.
Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Lang S, Kato M, Oefelein MG, Miyazono K, Kozlowski JM, Lee C. (1996b) Loss of expression of transforming growth factor-beta receptors type I and type II correlates with tumor grade in human prostate cancer tissues. Clinical Cancer Research 2:1255-1261.
Kim IY, Ahn HJ, Lang S, Oefelein MG, Oyasu R, Kozlowski JM, Lee C. (1998) Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients. Clinical Cancer Research 4:1625-1630.
Letterio JJ, Roberts AB. (1998) Regulation of immune responses by TGF-b. Annual Review Immunology 1998, 13:51-69.
Li AG, Lu SL, Han G, Kulesz-Martin M, Wang XJ. (2005) Current view of the role of transforming growth factor beta 1 in skin carcinogenesis. J Investig Dermatol Symp Proc 10:110-117.
Liu VC, Wong LW, Jang T, Shah AH, Park I, Yang X, Zhang Q, Lonning S, Teicher BA, Lee C. (2007) Tumor evasion of the immune system by converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T regulatory cells: role of tumor-derived TGF-b. Journal of Immunology 178:2883 2892.
Lu Q, Wu A, Richardson BC. (2005) Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. J Immunol 174:6212-6219.
Lu R, Wang X, Chen ZF, Sun DF, Tian XQ, Fang JY. (2007) Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells. *J Biol Chem* 282:12249-12259.
Luo X, Liu V, Zhang Q, Pothoven KL, Lee C. (2008) Cutting Edge: TGF-beta induced expression of Foxp3 in T cells is mediated through inactivation of ERK. J Immunol 180:2757-2761.
Massague J. (1990) The transforming growth factor b family. Ann Rev Cell Bio 6:597-641.
Massague J, Cheifetz S, Laiho M, Ralph DA, Weis F, Zentella A. (1992) TGF-beta. Cancer Surv 12:81-103.
McCormick LL, Zhang Y, Tootell E, Gilliam AC. (1999) Anti-TGF-b treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: A model for human scleroderma. J Immunol 163:5639-5699.
Nam JS, Terabe M, Mamura M, Kang MJ, Chae H, Stuelten C, Kohn E, Tang B, Sabzevari H, Anver MR, Lawrence S, Danielpour D, Lonning S, Berzofsky JA, Wakefield LM. An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Res. 2008 May 15;68(10):3835-43.
Oelke K, Richardson B. (2004) Decreased T cell ERK pathway signaling may contribute to the development of lupus through effects on DNA methylation and gene expression. Int Rev Immunol 23:315-331

Pardali K, Moustakas A. (2007) Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta 1775:21-62

Perry K, Wong L, Liu V, Park I, Zhang Q, Rejen V, Huang X, Smith ND, Jovanovic B, Lonning S, Teicher BA, Lee C. (2008) Treatment of transforming growth factor-beta (TGF-beta) insensitive mouse Renca tumor by TGF-beta elimination. Urology

Pinkas J, Teicher BA. (2006) TGF-beta in cancer and as a therapeutic target. Biochem Pharmacol 72:523-529.

Prud'homme GJ. (2007) Pathobiology of transforming growth factor beta in cancer, fibrosis and immunologic disease, and therapeutic considerations. Lab Invest 87:1077-1091.

Rosenbaum E, Hoque MO, Cohen Y, Zahurak M, Eisenberger MA, Epstein JI, Partin AW, Sidransky D. (2005) Promoter hypermethylation as an independent prognostic factor for relapse in patients with prostate cancer following radical prostatectomy. Clin Cancer Res 11:8321-8325.

Ruzek MC, Hawes M, Pratt B, McPherson J, Ledbetter S, Richards SM, Garman RD. (2003) Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. Immunopharmacol Immunotoxicol 25:235-257.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155:1151-1164.

Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. (2003) CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. Cancer 98:1089.

Selvaraj RK, Geiger TL. (2007) A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. J Immunol 178:7667-7677.

Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. (2002a) Reconstitution of lethally irradiated mice with TGF-b insensitive bone marrow leads to myeloid expansion and inflammatory disease. Journal of Immunology 169:3485-3491.

Shah AH, Tabayoyong WB, Kundu SD, Kim SJ, van Paris L, Liu VC, Kwon E, Greenberg NM, Lee C. (2002b) Suppression of tumor metastasis by blockade of TGF-b signaling in bone marrow cells through a retroviral mediated gene therapy in mice. Cancer Research 62:7135-7138.

Shimizu J, Yamazaki S, Sakaguchi S. (1999) Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 163:5211.

Wang XJ. (2001) Role of TGFbeta signaling in skin carcinogenesis. Microsc Res Tech 52:420-429.

Wojtowicz-Praga S. (1997) Reversal of tumor-induced immunosuppression: A new approach to cancer therapy. J Immunother 20:165-177.

Wojtowicz-Praga S. (2003) Reversal of tumor-induced immunpsuppresion by TGF-b inhibitors. *Invest New* Drugs 21:21-32.

Yu N, Kozlowski JM, Park II, Chen L, Zhang Q, Xu D, Doll JA, Crawford SE, Brendler CB, Lee C. (2010) Over-expression of transforming growth factor β1 in malignant prostate cells is partly caused by a runaway of TGF-β1 auto-induction mediated through a defective recruitment of protein phosphatase 2A by TGF-β type I receptor. Urology 76(6):1519.e8-13

Zeng L, Rowland RG, Lele SM, Kyprianou N. (2004) Apoptosis incidence and protein expression of p53, TGF-beta receptor II, p27Kip1, and Smad4 in benign, premalignant, and malignant human prostate. Hum Pathol 35:290-297.

Zhang Q, Yang X, Pins M, Liu V, Jovanovic B, Kuzel T, Kim S-J, Van Parijs L, Greenberg NM, Guo Y, Lee C. (2005a) Adoptive transfer of tumor reactive TGF-beta insensitive $CD8^+$ T cells: Eradication of autologous mouse prostate cancer. Cancer Research 65:1761-1769.

Zhang Q, Rubenstein JN, Liu VC, Park I, Jang T, Lee C. (2005b) Restoration of expression of transforming growth factor-beta type □□ receptor in murine renal cell carcinoma (Renca) cells by 5-Aza- -deoxycytidine. Life Sciences 76:1159-1166.

Zhang Q, Rubenstein JN, Jang TL, Yang X, Pins M, Jovanovic B, Kim SJ, Park I, Liu V, Lee C. (2005c) Insensitivity to transforming growth factor-beta signaling is resulted from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). Molecular Endocrinology 19:2390-2399.

Zhang Q, Helfand BT, Jang TL, Zhu LJ, Chen L, Yang XJ, Kozlowski J, Smith N, Kundu SD, Yang G, Raji AA, Javonovic B, Pins M, Lindholm P, Guo Y, Catalona WJ, Lee C. (2009) NF-kB-Mediated Transforming Growth Factor-β-Induced Expression of Vimentin is an Independent Predictor of Biochemical Recurrence After Radical Prostatectomy. Clinical Cancer Res 19:128-139.

Zhang Q, Chen L, Helfand BT, Zhu LJ, Kozlowski J, Minn A, Jang T, Yang XJ, Javonovic B, Guo Y, Lonning S, Harper J, Teicher BA, Yu N, Brendler C, Wang J, Catalona WJ, Lee C. (2011) Transforming Growth Factor-β-induced DNA methyltransferase contributes to aggressive prostate cancer phenotypes and predicts biochemical recurrence after radical prostatectomy. PLoS ONE 2011;6(9):e25168.

APPENDICES:

Yu N, Kozlowski JM, Park II, Chen L, Zhang Q, Xu D, Doll JA, Crawford SE, Brendler CB, Lee C. (2010) Over-expression of transforming growth factor β1 in malignant prostate cells is partly caused by a runaway of TGF-β1 auto-induction mediated through a defective recruitment of protein phosphatase 2A by TGF-β type I receptor. Urology 76(6):1519.e8-13

Zhang Q, Chen L, Helfand BT, Zhu LJ, Kozlowski J, Minn A, Jang T, Yang XJ, Javonovic B, Guo Y, Lonning S, Harper J, Teicher BA, Yu N, Brendler C, Wang J, Catalona WJ, Lee C. (2011) Transforming Growth Factor-β-induced DNA methyltransferase contributes to aggressive prostate cancer phenotypes and predicts biochemical recurrence after radical prostatectomy. PLoS ONE 6(9): e25168.

Zhang Q, Chen L, Helfand BT, Zhu LJ, Kozlowski J, Minn A, Jang T, Yang XJ, Javonovic B, Guo Y, Lonning S, Harper J, Teicher BA, Yu N, Brendler C, Wang J, Catalona WJ, Lee C. The recruitment of PP2A by TGF-β receptors mediates the response to T -β-induced activation of ERK in prostate cancer. 2011 102nd American Association of Cancer Research (AACR) Annual meeting, Orlando, FL. Cancer Research, 2011. LB-3.

Zhang Q, Chen L, Helfand BT, Zhu LJ, Kozlowski J, Minn A, Jang T, Yang XJ, Javonovic B, Guo Y, Lonning S, Harper J, Teicher BA, Yu N, Brendler C, Wang J, Catalona WJ, Lee C. Anti-transforming growth factor beta antibody 1D11 suppresses the invasion of human prostate cancer cells. 2011 106th American Urological Association (AUA) Annual Meeting, Washington DC. Journal of Urology, 2011. AB-1100877.

Chung Lee, Qiang Zhang, James Kozlowski, Charles Brendler, Marcelo B. Soares, Atreya Dash, Michael McClelland, Michael McClelland, Dan Mercola Natural products and transforming growth factor-beta (TGF-β) signaling in cancer development and progression. urrent ancer Drug Target. In press.

Overexpression of Transforming Growth Factor β1 in Malignant Prostate Cells is Partly Caused by a Runaway of TGF-β1 Auto-induction Mediated Through a Defective Recruitment of Protein Phosphatase 2A by TGF-β Type I Receptor

Nengwang Yu, James M. Kozlowski, Irwin I. Park, Lin Chen, Qiang Zhang, Danfeng Xu, Jennifer A. Doll, Susan E. Crawford, Charles B. Brendler, and Chung Lee

OBJECTIVES To elucidate the mechanism of transforming growth factor (TGF)–β1 overexpression in prostate cancer cells.

METHODS Malignant (PC3, DU145) and benign (RWPE1, BPH1) prostate epithelial cells were used. Phosphatase activity was measured using a commercial kit. Recruitment of the regulatory subunit, Bα, of protein phosphatase 2A (PP2A-Bα) by TGF-β type I receptor (TβRI) was monitored by coimmunoprecipitation. Blockade of TGF-β1 signaling in cells was accomplished either by using TGF-β-neutralizing monoclonal antibody or by transduction of a dominant negative TGF-β type II receptor retroviral vector.

RESULTS Basal levels of TGF-β1 in malignant cells were significantly higher than those in benign cells. Blockade of TGF-β signaling resulted in a significant decrease in TGF-β1 expression in malignant cells, but not in benign cells. Upon TGF-β1 treatment (10 ng/mL), TGF-β1 expression was increased in malignant cells, but not in benign cells. This differential TGF-β1 auto-induction between benign and malignant cells correlated with differential activation of extracellular signal-regulated kinase (ERK). Following TGF-β1 treatment, the activity of serine/threonine phosphatase and recruitment of PP2A-Bα by TβRI increased in benign cells, but not in malignant cells. Inhibition of PP2A in benign cells resulted in an increase in ERK activation and in TGF-β1 auto-induction after TGF-β1 (10 ng/mL) treatment.

CONCLUSIONS These results suggest that TGF-β1 overexpression in malignant cells is caused, at least in part, by a runaway of TGF-β1 auto-induction through ERK activation because of a defective recruitment of PP2A-Bα by TβRI. UROLOGY 76: 1519.e8–1519.e13, 2010. © 2010 Elsevier Inc.

Many cancer cells, including prostate cancer, are able to overexpress TGF-β1.[1] Aside from the growth inhibitory effect, TGF-β1 can also stimulate extracellular matrix production, promote angiogenesis, facilitate invasion, and suppress the host immune system.[2] Thus cancer cells may circumvent the suppressive effects of TGF-β1, especially for aggressive cancer cells. Therefore for these cancer cells, it is possible that approaches to disrupt the TGF-β1 overexpression may offer a strategy to suppress the aggressive phenotype. To achieve this goal, we must first understand the mechanism through which these tumor cells produce more TGF-β1 than their benign counterparts. Yet, little is known about the regulation of TGF-β1 overexpression. The most potent inducer of TGF-β1 is itself.[3] It has been shown that TGF-β1 can induce its own mRNA transcription and protein synthesis in various cells.[4-8]

The exact mechanism of TGF-β1 auto-induction is still not clear. Previous investigations showed that extra-

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cellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) are involved in TGF-β-induced AP-1complex, which contributes to TGF-β1 auto-induction.[5] Further, Smad3/Smad4 takes part in the TGF-β1 mRNA transcription,[9] whereas p38 pathway influences de novo synthesis of TGF-β1.[9] Among these pathways, ERK activation by TGF-β1 has been shown to be essential for TGF-β1 auto-induction.[10] However, the effect of TGF-β1 on ERK activation remains controversial and seems to be dependent on cellular context. Many studies described that TGF-β1 could activate ERK;[11-15] however others reported that TGF-β1 inactivated or had no effect on ERK.[16-20] Therefore it is reasonable to deduce that TGF-β1 auto-induction through ERK activation is also cellular context dependent. The aim of the present study is to elucidate whether there is a difference in TGF-β1 auto-induction between malignant and benign prostate epithelial cells and whether TGF-β1 auto-induction contributes to TGF-β1 overexpression in cancer cells.

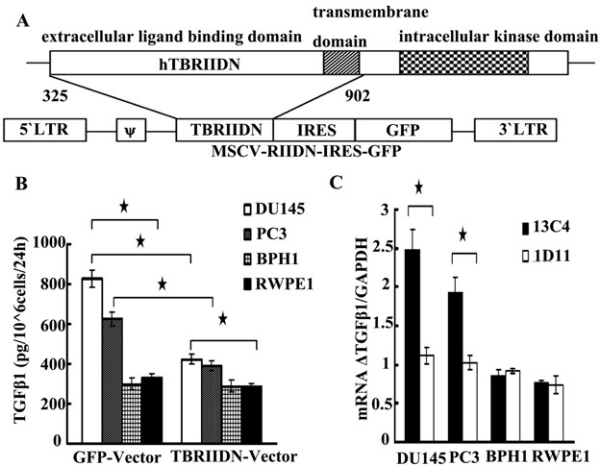

Figure 1. (A) schematic diagram of the murine stem cell virus (MSCV) retroviral construction. A truncated sequence of the human TβRIIDN, lacking the intracellular kinase signaling domain, was cloned into the pMig-internal ribosomal entry sequence (IRES)-GFP vector. The control construction (not shown) contained the GFP vector only and without the TβRIIDN sequences (325-902 bp). **(B)** cells infected with TβRIIDN or GFP vector were routinely cultured to 50% confluence and then rinsed with PBS thoroughly, cultured in serum-free medium for 24 more hours. TGF-β1 level in the culture medium was detected by using ELISA. **(C)** cells at 50% confluence were rinsed with PBS thoroughly and cultured in serum-free medium for 24 hours and then 100 μg/mL 13C4 or 1D11 was added. 12 h. later, total RNA was extracted and the ratio between TGF-β1 and GAPDH mRNA (mRNA ΔTGF-β1/GAPDH) was detected by qRT-PCR. Asterisks (*) denote that the *P* value is less than 0.05. Horizontal bars cover the groups that are being compared for statistical significance.

MATERIAL AND METHODS

Cell Lines, Reagents, and Retroviral Vector Transduction

Human prostate cancer cell lines, PC3, DU145, and the benign human prostate epithelial cell line, RWPE1, were obtained from American Type Culture Collection (Manassas, VA). Another human benign prostate epithelial cell line, BPH1, was kindly provided by Dr. Simon Hayward of Vanderbilt University. All cells, unless otherwise specified, were routinely maintained in culture medium RPMI-1640 with 10% fetal bovine serum (FBS) and kept in a 37°C, 5% CO_2 incubator. TGF-β1 was obtained from R&D Systems (Minneapolis, MN). The MEK1/2 inhibitor, UO126, was obtained from Cell Signaling (Danvers, MA). The TGF-β neutralizing monoclonal antibody (1D11) and the isotype control IgG Ab (13C4) were kindly provided by the Genzyme Corporation (Framingham, MA).

In indicated experiments, cells were transduced with TGF-β receptor II–dominant negative (TβRIIDN) retroviral vector or the GFP vector as previously described.[21,22] Figure 1A is a schematic diagram of the murine stem cell virus (MSCV) retroviral construct containing a truncated sequence of the human TβRIIDN, lacking the intracellular kinase signaling domain, which was cloned into the pMig-internal ribosomal entry sequence (IRES)-GFP vector. The control construct (not shown) contained the GFP vector only and without the TβRIIDN sequences (325-902 bp). The transduction efficiency was >90%.

Enzyme-Linked Immunosorbent Assay

Culture medium was collected and centrifuged at 1000 *g* for 10 minutes, and viable cells were counted. The concentration of TGF-β1 was detected by a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) following the manufacturer's instructions as described previously[22] (data expressed as pg/mL/10^6 cells/24 h).

Immunoprecipitation

Pierce Crosslink Immunoprecipitation kit (Pierce, Rockford, IL) was used for protein immunoprecipitation following the manufacturer's recommendations. Briefly, cells were harvested following the specified treatment with IP Lysis/Wash buffer plus 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was assayed and adjusted to 1 mg/mL with the lysis/wash buffer. An aliquot of 600 μL of cell lysates was precleared by using 20 μL Pierce Control Agarose Resin. TGF-β type I receptor (TβRI) antibody (5 μg) (Santa Cruz Biotechnology, CA) was bound to 20 μg of Pierce Plus Agarose in a Pierce Spin Column. After incubation for 60 minutes in room temperature, the antibody and agarose was crosslinked by DSS supplied by the kit. Precleared lysate was immunoprecipitated by the crosslinked antibody and agarose mixture for overnight on 4°C. Control agarose resin in the kit was used as a negative control when western-blot analysis was conducted.

Western Blot Analysis

Cell lysates were prepared by using cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with 1 mM PMSF and 1% protease inhibitor cocktail (Sigma-Aldrich) or eluted from the protein immunoprecipitation. Western blot analysis was performed as described previously.[18] The following antibodies were used: p-ERK1/2 (Cell Signaling, Danvers, MA), ERK1/2 (Cell Signaling), PP2A Bα antibody (Cell Signaling), Goat anti-mouse HRP-labeled secondary antibody (Bio-Rad Laboratories, Hercules, CA) and goat anti-rabbit HRP-labeled secondary antibody (Bio-Rad Laboratories).

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA) and digested with RNase free DNase I (Invitrogen) following the manufacturer' recommendations. cDNA was synthesized by using Superscript first-stand synthesis system (Invitrogen). Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed by using following primers specific for TGF-β1 (forward 5′-CCTTTCCTGCTTCTCATGGC-3′; reverse 5′-ACTTCCAGCCGAGGTCCTTG-3′), GAPDH (forward 5′-CAC CAC CAT GGA GAA GGC TGG-3′, reverse 5′-GAA GTC AGA GGA GAC CAC CTG-3′). The iQ SYBR Green Supermix (BioRad Laboratories) on iCycler iQ system was conducted according to the manufacturer's protocol. All qRT-PCR reactions were performed in triplicate for each cDNA sample.

Phosphatase Activity Assay

Nonradioactive Serine/Threonine Phosphatase Assay System (Promega, Madison, WI) was used according to the manufacturer's instructions. Cell lysates were prepared from 10^7 cells in 0.5 mL lysis buffer (10 mM Tris, pH 7.5; 0.1% Triton X-100; 140 mM NaCl; 1 mM PMSF; protease inhibitor cocktail) and passed through Sephadex G-25 columns to remove free phosphate. The protein concentration of the supernatant was determined (Pierce). The activity of the extract (corresponding to 2 μg protein) was measured in an enzyme-specific reaction buffer (250 mM imidazole pH 7.2; 1 mM EGTA, 0.1% β-mercaptoethanol; 0.5 mg/mL BSA) with 1 mM phosphopeptide and Molybdate Dye/Additive incubation. Results of colorimetric optical density (OD) were read at 620 nm. Calculations were performed from parallel measurements of standard free phosphate reactions. Similarly, protein-tyrosine-phosphatase activity was assayed by using the universal Tyrosine Phosphatase Assay Kit (Promega) according to the manufacturer's instructions.

Statistical Analysis

All experiments were repeated at least 3 times. Descriptive statistics (mean and standard deviation) was applied for data analysis. Analysis of variance test (multigroups) or Student's t test (2 groups) was applied for evaluating significance. A P value ≥ 0.05 was considered statistically significant.

RESULTS

Endogenous TGF-β1 and Its Auto-Induction

As indicated in Figure 1B, the basal level of TGF-β1 in DU145 and PC3 was significantly higher than that in BPH1 and RWPE1. These results verified the well-documented phenomenon that prostate cancer cells secrete more TGF-β1 than that of benign cells.[1] When cells were rendered insensitive to TGF-β1 by transduction with the TβRIIDN retroviral vector, the difference in TGF-β1 level between malignant and benign cells, although it still existed, was reduced when compared with that of the GFP vector controls. Following blockade of TGF-β1 signaling by TβRIIDN, the endogenous TGF-β1 level deceased significantly in DU145 and PC3, while the level did not change significantly in BPH1 and RWPE1. To validate the above result, we inhibited endogenous TGF-β1 by a neutralizing monoclonal antibody to TGF-β (1D11) for 12 hour and we determined the level of TGF-β1 mRNA. As 1D11 is able to neutralize all 3 TGF-β isoforms (TGF-β1, 2, 3), Fig. 1C showed that treatment with 1D11 resulted in a significant reduction in the basal level of TGF-β1 mRNA in malignant cells but not in benign cells. These results suggest that TGF-β1 auto-induction contributed, at least in part, to the high level of TGF-β1 expression in malignant cells, but there was no TGF-β1 auto-induction in benign cells under the basal condition.

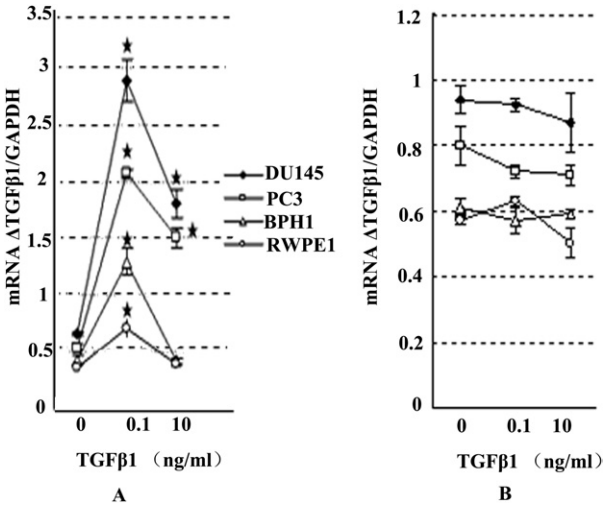

Figure 2. TGF-β1 mRNA transcription induced by exogenous TGF-β1. **(A)** cells at 50% confluence were rinsed with PBS thoroughly and cultured in serum-free medium for 12 hours with or without exogenous TGF-β1 and then TGF-β1 and GAPDH mRNA level was detected. **(B)** cells were treated as above, but 2 hours before TGF-β1 administration, 5 μM UO126 was added into the serum-free medium. ERK activation was inhibited thoroughly by this dose of UO126 (not shown). Asterisks (*) denote that the P value is less than 0.05 compared with the control.

Auto-Induction With Exogenous TGF-β1

Reports in the literature have shown that the phenomenon of TGF-β auto-induction with exogenous TGF-β1 in many cell types.[8] The results of the present study (Figure 2A) demonstrated that at a low dose (0.1 ng/mL) of exogenous TGF-β1, it induced TGF-β1 mRNA in both benign and malignant prostate cells; however, at a high dose (10 ng/mL), auto-induction occurred only in malignant cells. It was well documented that this increase in mRNA was consistent with an increase in de novo TGF-β1 synthesis.[8,9]

Impact of ERK Activation on TGF-β1 Auto-Induction

Following inhibition of ERK activation with UO126 (Figure 2B), auto-induction of TGF-β1 in these 4 cell lines were abrogated regardless the dosage of TGF-β1 used in the experiment. This observation was consistent with the study by Zhang et al, who reported that ERK

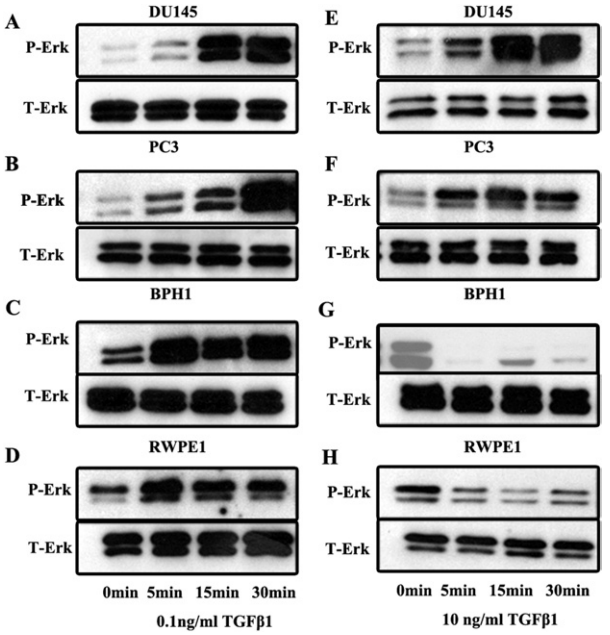

Figure 3. Western blot analysis of the effect of 0.1 ng/mL (left) or 10 ng/mL (right) TGF-β1 on expression of phosphorylated Erk (p-Erk), total Erk (T-Erk) in DU145 **(A** and **E)**, PC3 **(B** and **F)**, BPH1 **(C** and **G)**, and RWPE1 **(D** and **H)** over a period of 30 minutes. Cells at 50% confluence were starved with serum-free medium for overnight and then recovered with medium containing 10% fetal bovine serum for 2 hours before TGF-β1 treatment. Upon detection of p-Erk, the membrane was reprobed for T-Erk.

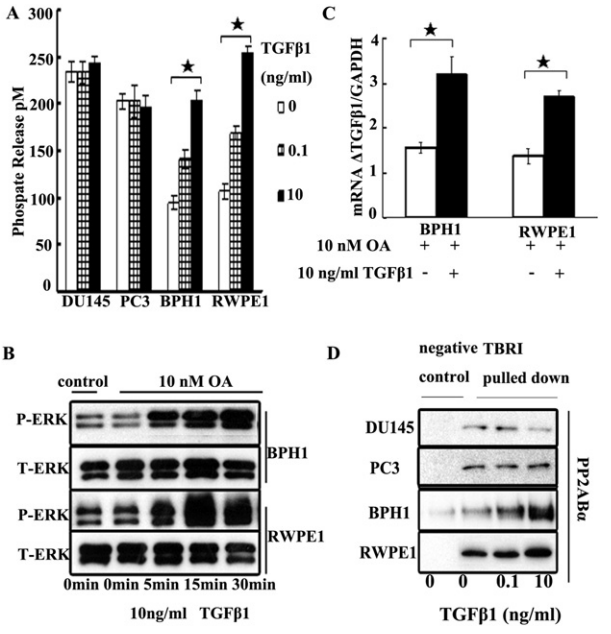

Figure 4. (A) effect of varying dosages of TGF-β1 (0, 0.1 and 10 ng/mL) on activity of protein serine/threonine phosphatases at 15 minutes following the TGF-β1 treatment. **(B)** okadaic acid (OA) (10 nM) was added 2 hours before TGF-β1 stimulation and then TGF-β-induced P-ERK change was detected in benign cells. To measure the impact of OA itself on P-ERK, cells without OA treatment were used as control. **(C)** Effect of OA on the high dose of TGF-β1 auto-induction in benign cells. OA was added 2 hours before TGF-β1 treatment and then cells were cultured for another 12 hours. **(D)** Effect of varying dosages of TGF-β1 on immunoprecipitation with antibody against TβRI. Reaction was stopped at 15 minutes after TGF-β1 treatment. PP2A-Bα was probed by western blot analysis in PC3, DU145, BPH1, and RWPE1 cells. Asterisks (*) denote that the *P* value is less than 0.05. Horizontal bars cover the groups that are being compared for statistical significance.

activation was linked to TGF-β1 auto-induction.[10] Interestingly, although following a low dose of TGF-β1 stimulation, a rapid ERK activation (p-ERK) was observed in both malignant and benign cells (Figure 3A-3D), at a high dose of TGF-β1, a rapid inactivation of ERK occurred in benign cells (Figure 3G and 3H) but the rapid ERK activation continued in malignant cells (Figure 3E and 3F). This differential activation of ERK between benign and malignant prostate epithelial cells coincided with the differential auto-induction of TGF-β1.

Mechanism for ERK Activation Change Induced by TGF-β1

It is known that TGF-β1 activates ERK through a direct phosphorylation of ShcA, which sets off the well characterized ShcA-Grb2-Sos-Ras-raf-Mek-ERK signal cascade.[11-14] Our results in benign cells suggested that, aside from the above positive pathway, there should also be a TGF-β1-mediated negative regulation of ERK activation. The possible candidate of this negative pathway is most likely protein phosphatase. There are mainly 2 classes of protein phosphatases: serine/threonine protein phosphatases and protein tyrosine phosphatases (PTP). When PTP was measured, the enzymatic activity did not change significantly in these 4 cell lines with TGF-β1 treatment (data not shown). However, when the serine/threonine phosphatase was measured, although there was no significant change in enzymatic activity in malignant cells, the phosphatase activity increased significantly in benign cells following TGF-β1 treatment (Figure 4A). This increase was in a dose-dependent manner. This finding coincided with previous study by Sebestyen et al.[20] Because PP2A is a main serine/threonine phosphatase and it is known that PP2A activity can be induced by TGF-β1, leading to the deactivation of ERK,[23,24] it is likely that PP2A may be the candidate phosphatase in the present system. Okadaic acid (OA) is an inhibitor of serine/threonine phosphatases, 10 nM of which will inhibit the PP2A but not sufficient to inhibit other phosphatases.[25] In the present study, treatment of OA (10 nM) to benign cells elicited the TGF-β1–mediated ERK activation at the high dose (10 ng/mL) of TGF-β1 (Figure 4B). Interestingly, following ERK activation, TGF-β1 auto-induction was observed (Figure 4C). This finding validated the observation that, in benign cells, TGF-β1 enhanced the PP2A activity, as reflected by the observed increase in serine/threonine phosphatase activity, resulting in ERK inactivation.

PP2A has 3 subunits: scaffold subunit A, regulatory subunit B, and catalytic subunit C. The PP2A core enzyme, consisting of A and C subunits, must interact with the regulatory subunit B to form a heterotrimeric holoenzyme to obtain the function of de-phoshorylation to the substrate. It is known that there is a physical interaction between the activated TβRI and PP2A-Bα, a WD-40 repeat regulatory subunit of PP2A and PP2A activity increases after this interaction.[26] To investigate whether there was physical interaction between PP2A-Bα and TβRI in these 4 cell lines, we performed an immunoprecipitation experiment using antibody to TβRI and probed for PP2A Bα in the precipitates by western blot analysis. Indeed, there was an increase in PP2A-Bα with TβRI pull-down following TGF-β1 treatment in a dose dependent manner in BPH1 and RWPE1 cells (Figure 4D). However, TGF-β1–induced PP2A Bα recruitment by TβRI was not observed in DU145 or PC3 cells, suggesting a defect in recruitment of PP2A Bα by TβRI. The total level of PP2A Bα protein in the total lysate of these 4 cell lines, as determined by western-blot analysis, was abundant and was not significantly different with different doses of TGF-β1 treatment (data not shown).

COMMENT

TGF-β1 is a pleiotropic cytokine that can mediate a wide spectrum of cellular effects through a variety of signaling pathways.[27] It is a well-known feature that sometimes different pathways cooperate to orchestrate a certain cellular effect. Here we demonstrate both ERK and PP2A play roles in TGF-β1 auto-induction in benign prostate cells, whereas in malignant prostate cells the TGF-β1 induced PP2A pathway was defective. Because the antibody used in this study detects the tyrosine phosphorylated site of ERK, ERK cannot be a direct substrate of PP2A here. Further studies are needed to investigate how PP2A exactly deactivates ERK.

As the physical and functional interaction between TβRI and PP2A Bα depends on the integrity and activation of both TβRI and TβRII,[26] although functional and somatic mutations of TβRI and TβRII in prostate cancer have been demonstrated before,[1] further studies should be conducted to determine whether the defective PP2A recruitment by TβRI in prostate malignant cells is the result of mutations in these receptors.

Although several hypotheses have been proposed to justify the high level of TGF-β1 overexpression in malignant cells,[2,28] a plausible mechanism remains elusive. In the present study, we observed a differential TGF-β1 signaling pathway between benign and malignant cells which leads to a differential auto-induction of TGF-β1 through a defective PP2A recruitment by TβRI in malignant cells. It should be pointed out that, under special conditions, such as low TGF-β1 microenvironment, benign cells can also mediate TGF-β1 auto-induction. Perhaps, this is a feedback mechanism in an effort to maintain homeostasis in benign cells. Once the level of TGF-β1 reaches a certain threshold in the benign environment, a sufficient level of PP2A will be recruited by TβRI, resulting in inactivation of ERK and termination of TGF-β1 auto-induction. However, in cancer cells, because of the defective recruitment of PP2A by TβRI, the auto-induction of TGF-β1 is runaway and constitutes a vicious cycle leading to overexpression of TGF-β1 and tumor progression. It is known that the average TGF-β1 in human serum[29] and semen[30] is higher than 10 ng/mL. The high dose of TGF-β1 used in the present study that induced an auto-induction of TGF-β1 in malignant cells but not in benign cells may have physiological significance. Further studies should be conducted to determine whether this defective recruitment of PP2A by TβRI is a widespread phenomenon among most malignant cells rather than limited to the 2 cancer cell lines used in the present study. If this phenomenon is validated in other malignant cells, it may offer a novel approach to prevent tumor invasion and tumor progression.

CONCLUSIONS

The present results indicate that a low level of TGF-β1 can auto-induce itself in both benign and malignant prostate epithelial cells. However, in benign cells, recruitment of PP2A by TβRI provides a mechanism to terminate the auto-induction at high dose of TGF-β1, while in malignant cells, because of a defective recruitment of PP2A by TβRI, TGF-β1 auto-induction is runaway, which contributes to the TGF-β1 overexpression in these cells.

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References

1. Lee C, Sintich SM, Mathews EP, et al. Transforming growth factor-beta in benign and malignant prostate. *Prostate*. 1999;39:285-290.
2. Massague J. TGFbeta in cancer. *Cell*. 2008;134:215-230.
3. Pardoux C, Derynck R. JNK regulates expression and autocrine signaling of TGF-beta1. *Mol Cell*. 2004;15:170-171.
4. Kelley J, Shull S, Walsh JJ, et al. Auto-induction of transforming growth factor-beta in human lung fibroblasts. *Am J Respir Cell Mol Biol*. 1993;8:417-424.
5. Kim SJ, Angel P, Lafyatis R, et al. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. *Mol Cell Biol*. 1990;10:1492-1497.
6. Li J, Tripathi BJ, Chalam KV, et al. Transforming growth factor-beta 1 and -beta 2 positively regulate TGF-beta 1 mRNA expression in trabecular cells. *Invest Ophthalmol Vis Sci*. 1996;37:2778-2782.
7. Lin RY, Sullivan KM, Argenta PA, et al. Exogenous transforming growth factor-beta amplifies its own expression and induces scar

formation in a model of human fetal skin repair. *Ann Surg*. 1995;222:146-154.
8. Van Obberghen-Schilling E, Roche NS, Flanders KC, et al. Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. *J Biol Chem*. 1988;263:7741-7746.
9. Annes J, Vassallo M, Munger JS, et al. A genetic screen to identify latent transforming growth factor beta activators. *Anal Biochem*. 2004;327:45-54.
10. Zhang M, Fraser D, Phillips A. ERK, p38, and Smad signaling pathways differentially regulate transforming growth factor-beta1 autoinduction in proximal tubular epithelial cells. *Am J Pathol*. 2006;169:1282-1293.
11. Aoki H, Ohnishi H, Hama K, et al. Autocrine loop between TGF-beta1 and IL-1beta through SmadIII- and ERK-dependent pathways in rat pancreatic stellate cells. *Am J Physiol Cell Physiol*. 2006;290:C1100-C1108.
12. Chen G, Khalil N. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res*. 2006;7:2.
13. Guo B, Inoki K, Isono M, et al. MAPK/AP-1-dependent regulation of PAI-1 gene expression by TGF-beta in rat mesangial cells. *Kidney Int*. 2005;68:972-984.
14. Lee MK, Pardoux C, Hall MC, et al. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J*. 2007;26:3957-3967.
15. Nickl-Jockschat T, Arslan F, Doerfelt A, et al. An imbalance between Smad and MAPK pathways is responsible for TGF-beta tumor promoting effects in high-grade gliomas. *Int J Oncol*. 2007; 30:499-507.
16. Dixon M, Agius L, Yeaman SJ, et al. Inhibition of rat hepatocyte proliferation by transforming growth factor beta and glucagon is associated with inhibition of ERK2 and p70 S6 kinase. *J Hepatol*. 1999;29:1418-1424.
17. Giehl K, Seidel B, Gierschik P, et al. TGFbeta1 represses proliferation of pancreatic carcinoma cells which correlates with Smad4-independent inhibition of ERK activation. *Oncogene*. 2000;19: 4531-4541.
18. Luo X, Zhang Q, Liu V, et al. Cutting edge: TGF-beta-induced expression of Foxp3 in T cells is mediated through inactivation of ERK. *J Immunol*. 2008;180:2757-2761.
19. Ramesh S, Qi XJ, Wildey GM, et al. TGF beta-mediated BIM expression and apoptosis are regulated through SMAD3-dependent expression of the MAPK phosphatase MKP2. *EMBO Rep*. 2008;9: 990-997.
20. Sebestyen A, Hajdu M, Kis L, et al. SmadIV-independent, PP2A-dependent apoptotic effect of exogenous transforming growth factor beta 1 in lymphoma cells. *Exp Cell Res*. 2007;313:3167-3174.
21. Shah AH, Tabayoyong WB, Kimm SY, et al. Reconstitution of lethally irradiated adult mice with dominant negative TGF-beta type II receptor-transduced bone marrow leads to myeloid expansion and inflammatory disease. *J Immunol*. 2002;169:3485-3491.
22. Zhang Q, Helfand BT, Jang TL, et al. Nuclear factor-kappaB-mediated transforming growth factor-beta-induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy. *Clin Cancer Res*. 2009;15:3557-3567.
23. Dent P, Jelinek T, Morrison DK, et al. Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. *Science*. 1995;268:1902-1906.
24. Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J*. 2008;22:954-965.
25. MacKintosh C, Beattie KA, Klumpp S, et al. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett*. 1990;264:187-192.
26. Griswold-Prenner I, Kamibayashi C, Maruoka EM, et al. Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. *Mol Cell Biol*;1998:6595-6604.
27. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature*. 2003;425: 577-584.
28. Hata A. TGFbeta signaling and cancer. *Exp Cell Res*. 2001;264: 111-116.
29. Wolff JM, Fandel T, Borchers H, et al. Transforming growth factor-beta1 serum concentration in patients with prostatic cancer and benign prostatic hyperplasia. *Br J Urol*. 1998;81:403-405.
30. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res*. 2005;322:43-52.

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TGF-β Regulates DNA Methyltransferase Expression in Prostate Cancer, Correlates with Aggressive Capabilities, and Predicts Disease Recurrence

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Abstract

Background: DNA methyltransferase (DNMT) is one of the major factors mediating the methylation of cancer related genes such as TGF-β receptors (TβRs). This in turn may result in a loss of sensitivity to physiologic levels of TGF-β in aggressive prostate cancer (CaP). The specific mechanisms of DNMT's role in CaP remain undetermined. In this study, we describe the mechanism of TGF-β-mediated DNMT in CaP and its association with clinical outcomes following radical prostatectomy.

Methodology/Principal Findings: We used human CaP cell lines with varying degrees of invasive capability to describe how TGF-β mediates the expression of DNMT in CaP, and its effects on methylation status of TGF-β receptors and the invasive capability of CaP in vitro and in vivo. Furthermore, we determined the association between DNMT expression and clinical outcome after radical prostatectomy. We found that more aggressive CaP cells had significantly higher TGF-β levels, increased expression of DNMT, but reduced TβRs when compared to benign prostate cells and less aggressive prostate cancer cells. Blockade of TGF-β signaling or ERK activation (p-ERK) was associated with a dramatic decrease in the expression of DNMT, which results in a coincident increase in the expression of TβRs. Blockade of either TGF-β signaling or DNMT dramatically decreased the invasive capabilities of CaP. Inhibition of TGF-β in an TRAMP-C2 CaP model in C57BL/6 mice using 1D11 was associated with downregulation of DNMTs and p-ERK and impairment in tumor growth. Finally, independent of Gleason grade, increased DNMT1 expression was associated with biochemical recurrence following surgical treatment for prostate cancer.

Conclusions and Significance: Our findings demonstrate that CaP derived TGF-β may induce the expression of DNMTs in CaP which is associated with methylation of its receptors and the aggressive potential of CaP. In addition, DNMTs is an independent predictor for disease recurrence after prostatectomy, and may have clinical implications for CaP prognostication and therapy.

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Introduction

TGF-β is a pleiotropic growth factor that has been implicated in multiple, and often diametrically opposed functions, including cell proliferation, cell growth arrest, differentiation, and apoptosis [1], [2]. An obvious question raised by these diverse functions is how TGF-β mediates these seemingly contradictory roles in both cancer and benign cells. In cancer cells, TGF-β acts as a growth promoter and aids in metastasis, whereas in normal cells it appears to inhibit cell growth and induce apoptosis [3]. Characteristics of aggressive prostate cancer (CaP) include a gradual loss of sensitivity to TGF-β and over-expression of TGF-β, which appears to initiate a vicious cycle for tumor progression. Although it is well known that a reduction or loss of expression of TGF-β receptors (TβRs) enables cancer cells to escape the growth inhibitory effect of TGF-β and to gain a growth advantage, the cellular mechanism(s) underlying these events in human CaP cells remains undefined. Previously, we have demonstrated that the loss of TβRs expression by promoter methylation is associated with insensitivity to TGF-β-mediated growth inhibition [4].

DNA methylation is carried out by DNA methyltransferases (DNMTs). There are at least three functional DNMTs that have been identified in eukaryotic systems. DNMT1 has been implicated primarily in the maintenance of methylation patterns that occurs during cellular replication, and it preferentially methylates hemi-methylated DNA [5]. It has been the most extensively studied maintenance methyltransferase and is abundant in tumor cells and tissues. In comparison, DNMT2 does not appear to have significant methylation activity and DNMT3L is likely to be limited to DNA methylation during germline development [5]. Finally, DNMT3A and DNMT3B are known to be de novo methylators of CpG sites [6], which have higher methyltransferase activity for unmethylated DNA than DNMT1 and can contribute to de novo methylation during embryogenesis [7], [8]. Although DNMT is reported to be associated with some aggressive cancers like hepatocellular carcinomas, stomach cancers, non-small cell lung cancers, lymphoma and prostate cancers [9], [10], [11], [12], [13], its role remains controversial and the overall regulation, coordination and activity of DNMTs is unclear with different cancers. Furthermore, the mechanism of DNMTs in cancer cells and its association with invasive malignant capabilities and clinical outcomes after treatment have not been described.

We recently reported that the epigenetic regulation of TGF-β-induced expression of Foxp3 may be mediated through the inactivation of extracellular signal-regulated kinases (ERK), which may down-regulate DNMTs in benign cells [14]. As stated above, CaP cells and tissue are insensitive to TGF-β-mediated growth inhibition and have promoter methylation patterns which decrease the expression of TβRs (TβRI and TβRII) [4], [15], [16]. Taken together, these results indicate that the insensitivity to TGF-β in some CaP cells is at least partly due to the promoter methylation of TβRs. These findings have led us to explore the following two hypotheses in the present study: 1) There may be crosstalk between tumor derived TGF-β and DNMTs which is related to methylation in cancer; 2) DNMTs may be closely associated with the prostate cancer progression and outcomes following radical prostatectomy. To our knowledge, this subject matter has yet to be reported.

The purpose of our study was several-fold. First, we sought to investigate the corresponding changes in DNMT and TβRs expression and ERK activation after treating CaP cells with varying degrees of invasive capability and benign prostate epithelial cells with TGF-β. Next, we examined the effect of a neutralizing TGF-β antibody on the expression of DNMTs and tumor growth in vivo using a xenograft model. Finally, we determined whether activation of DNMTs was associated with biochemical recurrence following radical prostatectomy.

Materials and Methods

(A detailed explanation is presented in Method S1)

Cell Lines

The mouse CaP cell line TRAMP-C2 cells was obtained from Dr. N. Greenberg [12]. The benign human prostate epithelial cell line, RWPE-1, was purchased from American type culture collection (ATCC). BPH-1 cells were kindly provided by Dr. Simon Hayward. Four variants of the human CaP PC-3 cell lines (PC-3, PC-3M-Pro4, PC-3M and PC-3M-LN4) with varying degrees of invasive capabilities were kindly provided by Dr. Fidler and Dr. Pettaway [17], [18], [19], [20]. The reason we chose PC-3 variants was because these variants originate from the same cell line, but vary in their aggressive capabilities. The results of signaling regulation were more comparable in contrast to using the different kinds of CaP cell lines. For some experiments, cells were rendered insensitive to TGF-β (as a negative control) by introducing a TβRIIDN as previously described [21], [22]. In some experiments, cells were treated with or without TGF-β1 or MEK inhibitor U0126 (Promega). Finally, some experiments involved the use of Anti-TGF-β (1, -2, -3) neutralizing mAb (clone 1D11; a gift from Genzyme Corporation) as previously described [23], [24]. (Method S1).

TGF-β1 ELISA

RWPE-1, BPH-1 and all PC3 variants and the corresponding TβRIIDN infected cell lines were cultured in fresh serum-free media for 24 hours. TGF-β1 ELISA was carried out using the Human TGF-β1 Immunoassay Kit (R&D Systems) (Method S1).

[^{3}H]-Thymidine Incorporation Assay

All cells were grown in culture for 48 hours. Cells were then exposed to a medium containing [^{3}H]-thymidine (0.5 μCi/mL; Amersham Biosciences) for an additional 5 hours. Thymidine incorporation was expressed as the fraction of counts found in cells of untreated controls (Method S1).

Western blot analysis

Western blot analyses were performed to compare TβRs, DNMTs and ERK expression after different treatments over time (Method S1).

Methylation-Specific PCR (MSP) and Sequencing

MSP for the methylation status of TβRs was performed according to our previous report [4]. The methylated sites in cytosine positions with/without treatment of 5-Aza or TGF-β were identified.

Immunofluorescence and Co-staining

Immunofluorescence studies were performed on all PC-3 cell line derivatives as previously described [22], [25]. For co-localization of DNMTs and phosphorylated ERK (p-ERK), cells were analyzed by using nucleus (DAPI)-DNMTs(TR)-p-ERK (FITC) triple staining (Method S1).

Quantitative RT-PCR

Human benign prostate epithelial cells RWPE-1 and BPH-1, and CaP PC-3 serials) were cultured in fresh media for 24 hours,

then exposed for 24 hours to either: 1) external recombinant TGF-β1 (10 ng/ml), 2) anti-TGF-β neutralizing monoclonal Ab (1D11; 5 μg/ml), or 3) MEK inhibitor U0126 (5 μM). Total RNA was extracted using an RNeasy kit (Qiagen). Primers for human for DNMTs [26] and TβRs [4] were listed in Method S1.

Cell invasion assay

Cell invasion assay (Matrigel invasion assay) was done in a 24-well Transwell chamber (8 μm pore size; CytoSelect; Cell Biolabs). Cells were plated at a density of 0.5×10^6 to 1.0×10^6/mL in serum-free medium. TGF-β1 and/or Erk inhibitor UO126, 5-Aza were added directly to the cell suspension, and 24 h later, the suspension was aspirated and the invaded cells were counted with a light microscope under high magnification objective (×100; Olympus) and measured at A560 nm in a plate reader after treatment with the extraction solution.

Animal Studies

The study was initiated using the subcutaneous (sc) injection of mouse prostate cancer TRAMP-C2 cells transfected with HSV1-tk-GFP-luciferase (SFG-nTGL) reporter gene expression vector [27], [28] into the right flank region of 30 C57BL/6 mice as described earlier [25]. Animals were randomly assigned to one of three groups following intraperitoneal injections with the specific anti-TGF-β neutralizing antibody 1D11 or control antibody 13C4 as described before [23,24]. All the mice were sacrificed after 15 injections of antibodies and group 3 were sacrificed on the same time interval. (Method S1). This study received approval from the institutional review board of Northwestern University (Evanston, IL). Northwestern University ACUC Approval protocol number 2007-0565." (Letter S1).

Construction of Tissue Microarrays (TMAs) and Clinical Outcome Assement

The existing clinical case information and banked tissue established within our prostate SPORE program database at Northwestern University was used. All enrolled subjects provided written informed consent by Northwestern Memorial Hospital and the study was approved by the Northwestern University Institutional Review Board (The IRB number is 1480-002, Letter S2). A total of 243 radical prostatectomy specimens were available with associated clinical information. A series of prostate TMAs were constructed with formalin-fixed, paraffin-embedded radical prostatectomy specimens as described previously [4], (Method S1 and Method S2).

Immunohistochemistry

All antibodies raised against DNMTs, phosphorylated ERK (p-ERK), total ERK (t-ERK), phosphorylated Smad2, TβRI and TβRII were first tested and optimized on whole-tissue sections and test arrays as previously described [4], [17], [29], [30], (Method S1 and Method S2).

Statistical Analysis. The SPSS 10.0.7 software package (SPSS, Inc.) was used for all analyses. Kaplan-Meier survival curve was analyzed by the log-rank test using the Graphpad Prism 4.02 software (Graphpad Software) (Method S1).

Results

1. DNMTs expression is associated with down regulation of TβRs and more invasive prostate cancer phenotypes

An ELISA assay was initially performed to determine whether there were differences in the endogenous expression levels of TGF-β in different CaP cell lines when compared to benign prostate cell lines. We found that all PC-3 cell lines expressed significantly higher levels of TGF-β (×2 to 6 times) compared to the BPH-1 and RWPE-1 ($p<0.05$). Furthermore, we found that more invasive cells (PC-3M and PC-3M-LN4) secreted almost 2 times higher baseline levels of TGF-β1 when compared with the less invasive cell lines (PC-3 and PC-3M-Pro4) (Fig. 1A).

We confirmed that different prostate cell lines behave differently in response to exogenous TGF-β1 exposure. For example, we found that RWPE-1 and BPH-1 cells were most sensitive to exogenous TGF-β1 as their growth was inhibited by 64.1% and 61.9%, respectively, after 24 hours of treatment with TGF-β1. In comparison, PC-3 and PC-3M-Pro4 cells were only inhibited by 13.7% and 12.3%, respectively. Finally, the growth rate of PC-3M-LN4 and PC-3M was unaffected by TGF-β1 exposure (Fig. 1B). Interestingly, in CaP cell lines, inhibition of TGF-β signaling, using the dominant negative type II TGF-β receptor (TßRIIDN) construct, was associated with significantly higher endogenous TβRII expression (using antibodies directed against the intracellular domain, because TβRIIDN includes only extracellular and transmembrane domains, but not intracellular domain) (Fig. 1C) and higher TGF-β secretion (Fig. 1D). In comparison, there was no difference in the expression of TGF-β in BPH-1 or RWPE-1 when they were infected with the retroviral TβRIIDN construct (Table S1).

In contrast to the expression of TGF-β, both TβRI and TβRII expression was significantly reduced in the more invasive cell lines, PC-3M-LN04 and PC-3M, compared with PC-3 and PC-3M-Pro4 cells (Fig. 2A). Blockade of TGF-β signaling with the TβRIIDN vector caused an approximately two to ten-fold increase in the expression of both TβRI and TβRII in all CaP cell lines (Fig. 2B). Taken together this suggests that increased baseline levels of TGF-β are associated with the inhibition of TβRs expression. Blockade of intracellular TGF-β signaling resulted in up-regulation of secretion of TGF-β in cancer cells.

Since promoter methylation of TβRs is associated with decreased expression [4], we compared the expression levels of DNMTs in the different CaP cell lines. In general, the more invasive PC-3M-LN4 and PC-3M cells showed an increased expression of DNMTs, when compared to the less invasive PC-3 and PC-3M-Pro4 (Fig. 2C). Blockade of TGF-β signaling with the TβRIIDN vector caused a ≥3-fold decrease in the expression of DNMTs in all CaP cell lines (Fig. 2D), and there was a corresponding increase in the expression of both TβRI and TβRII (Fig. 2B). The corresponding value (relative ratio of TβRs/GAPDH, or DNMTs/GAPDH) is shown in right panels. This finding was also supported by additional confirmatory studies. Immunoblot analyses demonstrated that after treatment with 5-Aza-2′-deoxycytidine (5-Aza), the expression of TβRI and TβRII in PC-3 increased dramatically. In contrast, the expression of both TβRI and TβRII decreased significantly with the treatment of TGF-β and this change could be recovered when 5-Aza is added (Figure S1A). Similarly, real-time PCR confirmed that the expression of both TβRI and TβRII was increased 2 to 2.5 folds after treatment of 5-Aza in PC-3 cells. Treatment with TGF-β suppressed the expressions of TβRI and TβRII 46% and 29% respectively (Figure S1B). We also identified the methylation status of TβRI and TβRII promoters, by using the same MSP approach and sequencing methodologies [4]. Using this technique, we found the same methylated sites as our previous study [4] in that cytosine positions −251, −231, −244, −348, −356 and −365 in the promoter of TβRI, and +27, +32 and −140 for the promoter of TβRII were methylated (Figure S1C). PC-3 cells also have a portion of TβRI and TβRII promoters that are unmethylated.

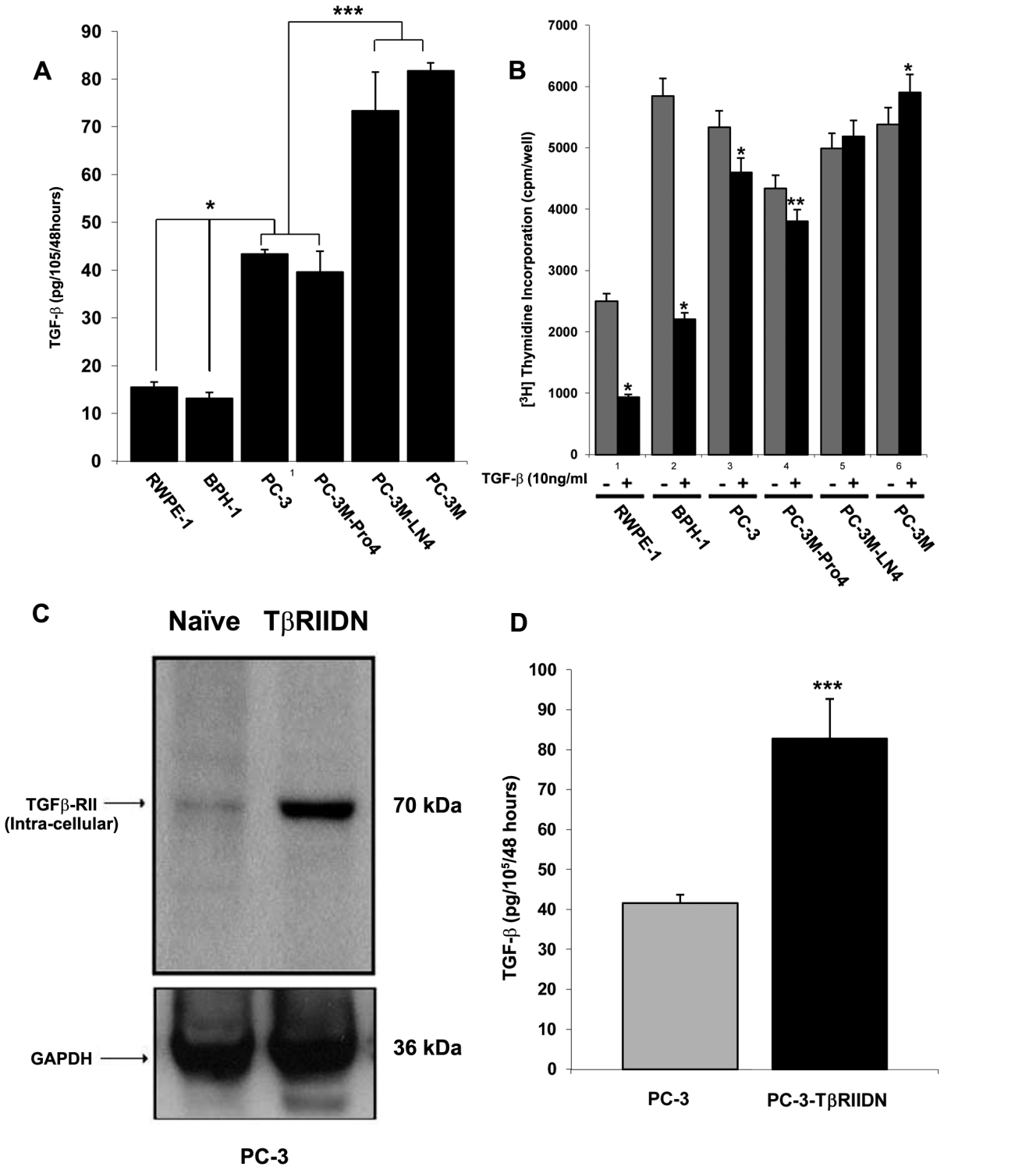

Figure 1. Tumor derived TGF-β regulates the expression of TβRs and secretion of TGF-β. A. ELISA assay demonstrating that PC-3 cell lines express significantly ($p<0.05$) higher (×2–6 times) levels of TGF-β compared to benign prostate cell lines, BPH-1 and RWPE-1. Furthermore, PC-3M and PC-3M-LN4, secreted almost 2 times higher baseline levels of TGF-β1 compared to PC-3 and PC-3M-Pro4 cells, respectively, which are less invasive. **B.** A thymidine incorporation assay indicates that the growth of RWPE-1 and BPH-1 cells is inhibited significantly by exposure to TGF-β1. In comparison, the growth of PC-3 and PC-3M-Pro4 cells is only slightly inhibited, and PC-3M-LN4 and PC-3M cells show no significant response to TGF-β1 exposure. **C.** In all cancer cell lines (here we show PC-3 as an example), inhibition of TGF-β using the TβRIIDN construct results in significantly higher naïve TβRII expression, and **D.** higher TGF-β secretion. Similar findings are found in the findings in the more invasive cell lines. In comparison, there was no difference in the expression of TβRII in BPH-1 or RWPE-1 when they were infected with TßRIIDN (Table S1).
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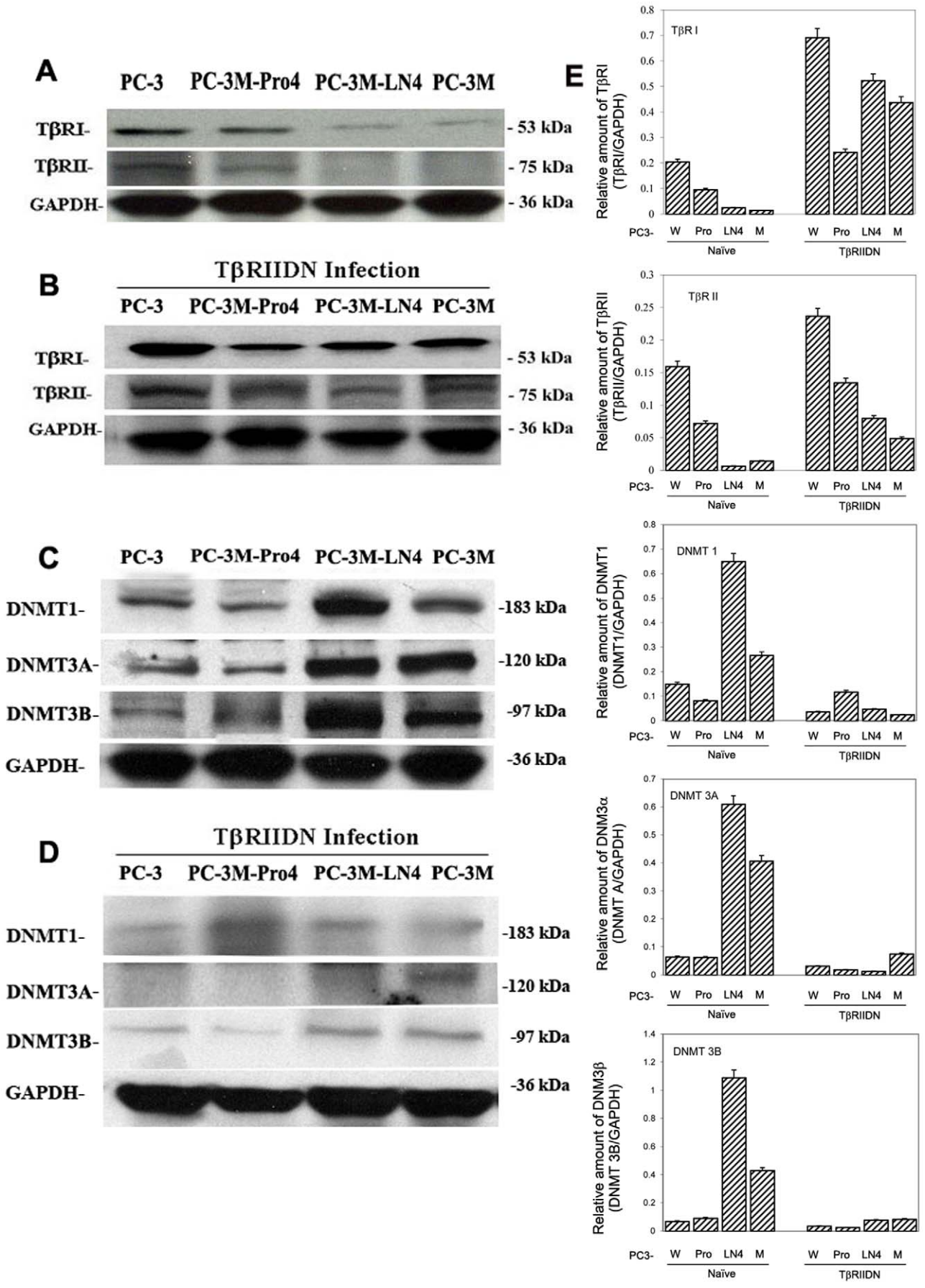
A
PC-3
PC-3M-Pro4
PC-3M-LN4
PC-3M
TβRI-
TβRII-
GAPDH-
- 53 kDa
- 75 kDa
- 36 kDa
B
TβRIIDN Infection
PC-3
PC-3M-Pro4
PC-3M-LN4
PC-3M
TβRI-
TβRII-
GAPDH-
- 53 kDa
- 75 kDa
- 36 kDa
C
PC-3
PC-3M-Pro4
PC-3M-LN4
PC-3M
DNMT1-
DNMT3A-
DNMT3B-
GAPDH-
-183 kDa
-120 kDa
-97 kDa
-36 kDa
D
TβRIIDN Infection
PC-3
PC-3M-Pro4
PC-3M-LN4
PC-3M
DNMT1-
DNMT3A-
DNMT3B-
GAPDH-
-183 kDa
-120 kDa
-97 kDa
-36 kDa
E
TβR I
Relative amount of TβRI (TβRI/GAPDH)
TβR II
Relative amount of TβRII (TβRII/GAPDH)
DNMT 1
Relative amount of DNMT1 (DNMT1/GAPDH)
DNMT 3A
Relative amount of DNM3α (DNMT A/GAPDH)
DNMT 3B
Relative amount of DNM3β (DNMT 3B/GAPDH)
PC3-
W
Pro
LN4
M
Naïve
TβRIIDN

Figure 2. TGF-β induced expression of DNMTs is inversely associated with the expression of TβRs. A. Western blot analyses demonstrate that in contrast to the expression of TGF-β, both TβRI and TβRII expression (as described in Figure 1B) is significantly reduced in the more invasive cell lines compared with less invasive cell lines. **B.** Blockade of TGF-β signaling with the TβRIIDN causes significant increase in the expression TβRIs in all cell lines. **C.** In contrast to the expression of TβRs, the over expression of DNMTs is associated with more invasive cell lines compared with the less invasive cell lines. **D.** Blockade of TGF-β signaling with TβRIIDN caused a ≥3-fold decrease in the expression of DNMTs. The corresponding value (relative ratio of TβRs/GAPDH, or DNMTs/GAPDH) is shown in right charts. (Fig. 2A and 2B were from the same Western Blot image, and Fig. 2C and 2D were from the other single Western blot image).
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Interestingly, treatment with TGF-β increased the methylation status, but treatment with 5-Aza converted all methylated sites to unmethylated. The thymidine incorporation assay indicated that the proliferation of PC-3 cells were only modestly inhibited modestly by exogenous TGF-β. In comparison, 5-Aza treatment resulted in significant inhibition of cell proliferation, regardless of whether exogenous TGF-β was added into the culture or not. There was no significant difference observed between treatment with both 5-Aza and TGF-β or with 5-Aza alone ($P>0.05$) (Figure S1D).

2. DNMTs expression is mediated through a phosphorylated-ERK dependent pathway

Our previous studies demonstrate that ERK may influence DNMT expression in benign cells [14]. We therefore sought to determine whether the level of activated ERK (phosphorylated ERK; p-ERK) is related to TGF-β-induced expression of DNMTs. To test this hypothesis, we first determined the level of p-ERK in benign prostate cells and compared it to the levels in different CaP cell lines. BPH-1 and RPWE-1 cells expressed significantly higher baseline levels of p-ERK than PC-3 cells (Fig. 3A). Interestingly, the time course of p-ERK expression after exposure to TGF-β was different between the benign and malignant cell lines. Specifically, there was a time dependent positive correlation between treatment with TGF-β1 and the expression of p-ERK in all PC-3 cell lines. In fact, this rapid increase in p-ERK expression (4-fold) began within 5 minutes following TGF-β1 treatment. The levels of p-ERK continued to increase during all subsequent time points up to 30 minutes after TGF-β1 addition. In contrast, the expression of p-ERK was rapidly inhibited (<5 minutes) after TGF-β1 addition to the media of benign cells, in a fashion that was independent of the total ERK protein expression (Fig. 3A). Immunofluorescence studies were subsequently used to help determine whether p-ERK and DNMTs were co-localized to the same cellular regions. To this end, confocal microscopic analyses of formaldehyde fixed immuno-stained PC-3 cells, in the absence or presence of TGF-β1, demonstrated co-loczalization between p-ERK and DNMTs signals. Only the cells with p-ERK immunofluorescence exhibited DNMT expression. In contrast, when PC-3 cells were rendered insensitive to TGF-β1 by transfection with the TβRIIDN, levels of both p-ERK and DNMTs were reduced dramatically as determined by immunofluorsence staining (Fig. 3B). To better quantify this relationship between TGF-β1, p-ERK and DNMTs, we next used real time PCR. These results demonstrated that exposure to TGF-β1 for 24 hours significantly increased the expression of all three DNMTs (~16.7%–14%) in all PC-3 cell lines studied. Treatment with an antibody specific for TGF-β1 (1D11; 5 mg/ml) or the specific ERK inhibitor, UO126, led to significant down-regulation of DNMTs mRNA expression (~33.9%–52.3%, and ~41.5%–57.6% respectively, Fig. 3C). These results suggest that TGF-β mediated expression of DNMTs is associated with an increase in p-ERK in cancer cells. Specifically, tumor derived TGF-β appears to be responsible for this ERK activation, as blockade of the original secreted TGF-β resulted in a great change in the expression of DNMTs (Fig. 3C). These results also suggest that tumor derived TGF-β mediated ERK activation is at least one of the major mediators for TGF-β induced expression of DNMTs which lead to TβRs down-regulation by promoter methylation in CaP [4], [14]. After treatment with TGF-β, there was a significant increase in the invasive capabilities of CaP cells. Invasion of CaP cells was inhibited by either TGF-β inhibitor 1D11, or p-Erk inhibitor U0126 or DNMT inhibor 5-Aza. The inhibition of invasion by the U0126 could not be reversed by TGF-β1 treatment. Importantly, DNMTs inhibitor 5-Aza can dramatically inhibited the CaP cells invasion, even more than blockade of TGF-β or p-ERK (Fig. 3D). This observation suggested that p-ERK was downstream factor of TGF-β, and synergistically mediates TGF-β regulated DNMTs which was closely associated with the invasive capability of CaP cells.

3. In vivo validation of the effects of TGF-β on ERK activation, DNMT expression, and prostate cancer growth

To validate whether TGF-β is responsible for the activation of ERK and up-regulation of DNMTs which may be involved in tumor progression in vivo, we conducted experiments using a mouse xenograft CaP model which involved the injection of CaP tumor cells (TRAMP-C2 cells stably transfected with a HSV1-tk-GFP-luciferase reporter, 5×10^6/each mouse). Tumor growth was followed using luciferase imaging. We used three groups of mice to better understand the effects of TGF-β on ERK activation and DNMT expression: Group 1: mice ($n=10$) received regular injections of the TGF-β neutralizing antibody, 1D11. Group 2: mice ($n=10$) received the isotype control antibody, 13C4, at the same regular intervals as Group 1. Group 3: received no treatment after xenograft injection as a control. We found that tumor growth was significantly inhibited with anti-TGF-β 1D11 antibody, treatment (Group 1) compared with the other two groups (Fig. 4A, 4B). In fact, at the end of the 45-day treatment period, one of the ten mice (10%) in this group was free of tumor. In the remaining 9 mice, the average tumor weight and volume was 5.3 g and 6.85 cm^3, respectively. In comparison, tumors were found in all mice in Groups 2 and 3. The average weight and volume of tumors in the 10 animals treated with the control antibody (Group 2) or no treatment (Group 3) was significantly greater (Fig. 4C). There were no metastases in all the groups as assessed by bioluminescence imaging. Immunohistochemical analyses of the primary tumors revealed that the expression of p-ERK and DNMTs in animals in Group 1 were significantly lower than those of the other two groups (Fig. 4D).

4. DNMTs correlates with clinical characteristics

To evaluate the association between TGF-β and the induction of DNMTs in CaP specimens, we compared the expression levels of TGF-β1, ERK, p-ERK, TβRI, TβRII, p-Smad2, and DNMTs in archived tissue microarray specimens obtained at the time of radical prostatectomy and correlated them with corresponding patients' clinical and pathologic characteristics (Table S2. Each marker was assigned a value of 0 (<20% cell immunostaining), 1

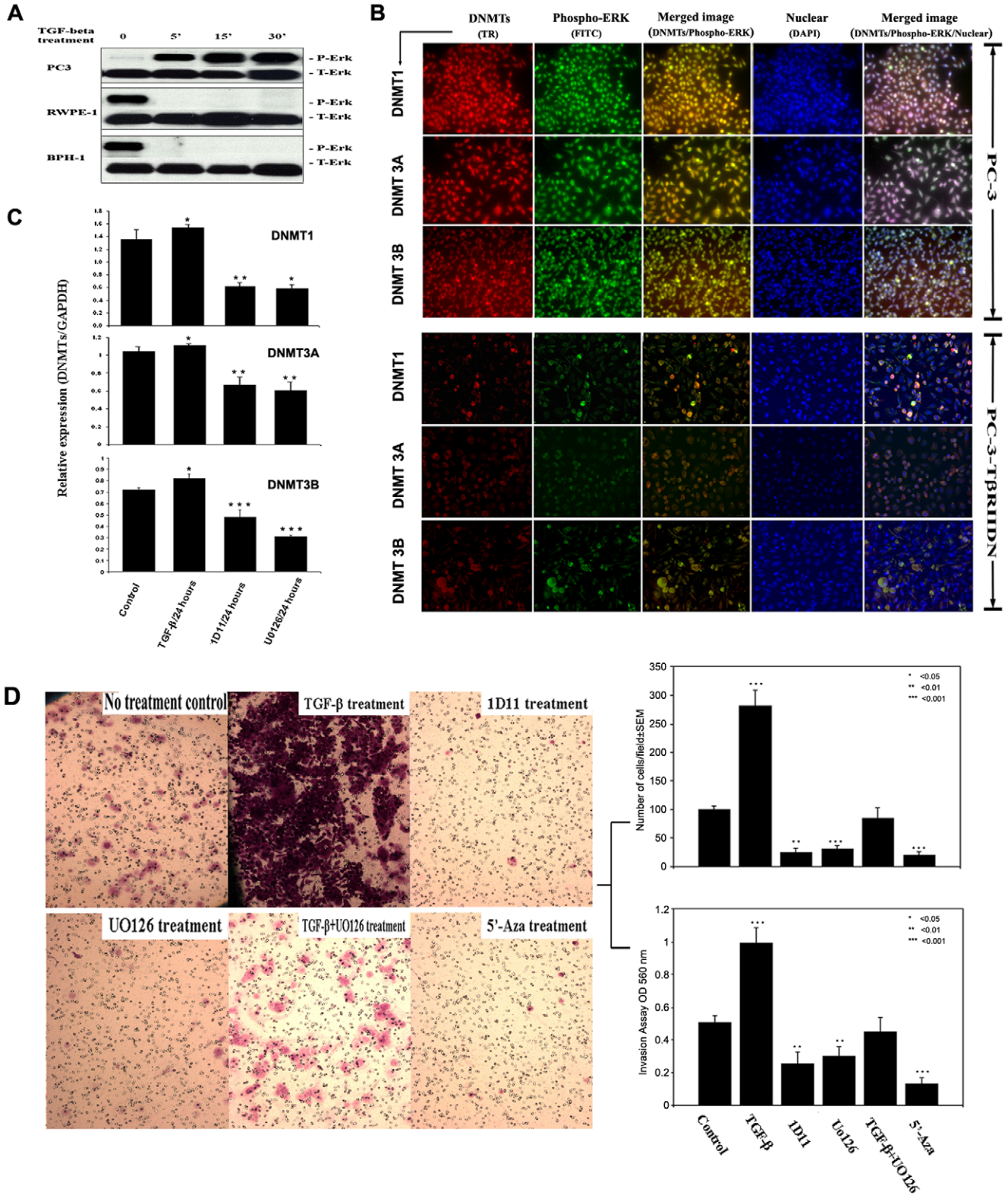

Figure 3. TGF-β induced DNMTs is mediated by ERK activation. A. The benign BPH-1 and RPWE-1 cells express significantly higher baseline levels of p-ERK than the PC-3 cells. There is a time dependent positive correlation between treatment with TGF-β1 and the expression of p-ERK in PC-3 cells. The levels of p-ERK continue to increase during all subsequent time points up to 30 minutes after TGF-β1 addition. In contrast, the expression of p-ERK is rapidly (<5 minutes) inhibited after TGF-β1 exposure in benign cells in a fashion that is independent of the total ERK protein expression. **B.** Immunofluorescence reveals that only cells (this is PC3 for example) expressing p-ERK exhibit DNMT expression. In contrast, when PC-3 cells are rendered insensitive to TGF-ß1 by TβRIIDN, levels of both p-ERK and DNMT are significantly reduced (magnification: 10×20). **C.** We performed real time PCR to better quantify the relationship between TGF-β1, p-ERK and DNMTs. Exposure to TGF-β1 significantly increased the expression of all

three DNMTs in PC-3 cells. Treatment with 1D11, or MEK inhibitor, UO126 is associated with the down-regulation of all DNMT mRNA expression. **D.** (Here we showed most aggressive PC-3M as a sample). There was a significant increase in cell motility through a Matrigel-coated polycarbonate membrane under the treatment of TGF-β1 (10 ng/mL). The invasion of all CaP cells could be inhibited by blocking the TGF-β signal by 1D11 or using a p-ERK inhibitor UO126, or DNMT inhibitor 5-Aza separately. The inhibition of invasion by UO126 can't be reverted by TGF-β treatment. Upper right panel: Corresponding numbers of invasive cells. Bottom right panel: absorbance values. This result indicates p-ERK mediated TGF-β-induced DNMT potentiates the invasive ability of prostate cancer cell lines. (magnification, 10×10).
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(20–49% cell immunostaining), 2 (50–74% cell immunostaining) and 3 (75–100% cell immunostaining) depending on the percentage of cancer cells showing positive immunostaining. The positive and negative control staining was showed in the "Figure S2". We found that a high level of expression of TGF-β1, p-ERK and DNMTs coupled with a low level of expression of TβRI, TβRII, and p-Smad2 was associated with adverse pathologic features, such as higher Gleason's grade (Fig. 5A, Fig. 5B, Table S3). These results correspond to our finding in PC-3M-LN4 and PC-3M cells that TGF-β induced DNMTs are associated with clinically more aggressive phenotypes.

We found a significant correlation between the expression of TGF-β1 and DNMTs in these tissue microarray specimens. There was also a significant correlation between TGF-β and p-ERK, TGF-β and TβRI, p-ERK and DNMT1, p-ERK and DNMT3A, p-ERK and DNMT3B respectively. In addition, we found a significant correlation between the expression levels of all three of the DNMTs. There were inverse relationships between DNMTs and TβRs, DNMT1.vs. TβRI, DNMT1.vs. TβRII, DNMT3A vs. TβRII (Table S3).

5. DNMTs is associated with biochemical recurrence in prostate cancer patients after radical prostatectomy

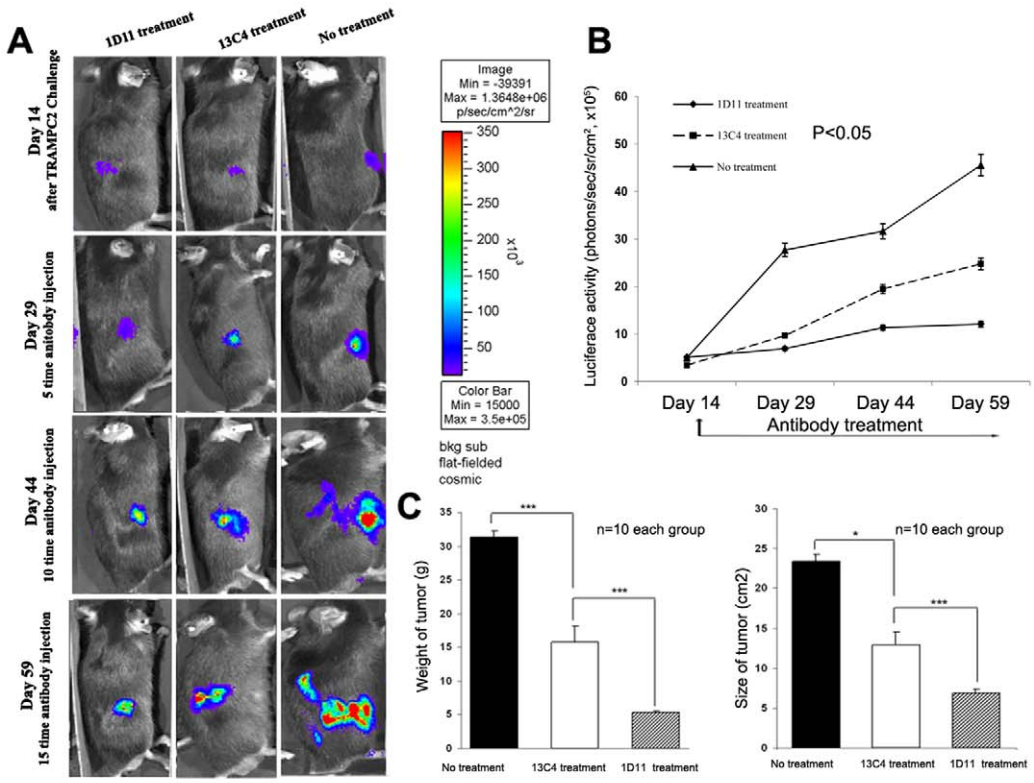
To examine the utility of these markers as possible prognostic tools, we correlated the expression levels of the above TGF-β related biomarkers of each tumor with the clinical outcome of the corresponding patient using the database of Northwestern University's Prostate SPORE. The log rank test was used determine whether or not these various markers correlated with biochemical recurrence (PSA>0.2 ng/ml after radical prostatectomy). Variables of interest included all TMA markers, clinical stage, clinical Gleason's score, which was grouped as 4–6, 7, 8–10, surgical margin status, PSA doubling time, and patient age. As mentioned above, all specimens were assigned a value between 0–3 based upon the percentage of cancer cells showing a positive staining. A Kaplan Meier curve was generated for each of the above significant variables.

Expression levels of TGF-β1, p-Smad2, p-ERK, pathologic Gleason Score and DNMT1, TβRI were associated with biochemical recurrence after radical prostatectomy (Fig. 6A. B, Table S4). The degree of DNMT1 expression correlated significantly with biochemical recurrence [$P = 0.0043$ using 4 expression groups; (0, 1, 2, 3; $P = 4\times10^{-04}$ using 2 groups; low expression (0, 1, 2) vs high expression(3)]. DNMT3A and DNMT3B, surgical margin status, TGF-β type II receptor expression level and PSA doubling time were not associated with biochemical recurrence ($p>0.05$). To determine the best model for predicting PSA recurrence, a Cox Proportional Hazards Model was fit to include all the significant variables and backward selection method was used to eliminate non-significant variables. The final selected model includes DNMT1, grouped as below 3 (low expression) or above 3 (high expression; log Rank $P = 0.002$; hazard ratio = 3. 53; 95% CI 1.6–7.78), and pathologic Gleason score sum of patients, grouped as below 8, or above (log Rank $P = 0.034$; hazard ratio = 2.27; 95% CI 1. 06–4.83) (Fig. 6C, Table S5). Patients whose tumors had a DNMT1 expression level of 3 (high expression) had a 3.53 higher risk of recurrence than patients with lower scores of DNMT1 in the tumor. Even in patients with low Gleason grade (≤6), there was a high risk of recurrence if high levels of DNMT1 expression were present. A high DNMT1 expression was independently associated with biochemical recurrence, irrespective of Gleason score. There was no correlation between PSA doubling time and the expression levels of DNMT1.

Discussion

Many malignancies, including CaP, exhibit aberrant methylation within the promoter regions of genes associated with a loss of function [31], [32], [33]. Presumably, this loss of function contributes to the development and progression of the disease. DNMTs are the major mediators responsible for the hypermethylation of the promoter regions of many genes encoding for signaling factors including the TβRs promoter [4], which may subsequently inhibits TβRs translation which ultimately results in the insensitivity to the normal inhibitory effects of TGF-β, uninhibited growth and progression of cancer [4], [34], [35], [36]. Although DNMTs are recognized as important regulators of transcription of carcinogenesis [37], [38], [39], [40], [41], [42], and have been a topic of considerable interest in the last few years, their assessment in vivo and within human specimens remains uncertain. Our study findings demonstrate that high level of expression of DNMTs is associated with more aggressive phenotypes of CaP, lower expression of TβRs, and lower sensitivity to the inhibitory role of TGF-β.

The molecular mechanisms which govern regulation of DNMTs have been largely unknown [43], and the relationship(s) between DNMTs and TGF-β in CaP have yet to be explored. Although other factors like c-Jun may be involved in the process [44], ERK appears to be an obligatory switch for TGF-β-mediated expression of DNMTs in CaP, although the effect of TGF-β on ERK activation remains controversial [45], [46]. More recently we reported that there was a differential activation of ERK between benign and malignant cells in response to TGF-β [14], [47]. In our prior studies involving benign cells, we reported that TGF-β exposure, ERK inactivation and DNMTs down regulation contribute to the expression of Foxp3 in benign immune cells [14]. In the present study, higher expression levels of DNMTs were found to be associated with CaP with higher invasive capabilities when compared with CaP cells with lower invasive capabilities. Interestingly, we found that increased levels of DNMTs were associated with increased levels of TGF-β and p-ERK, and decreased levels of TβRs. In contrast, our hypotheses were verified by a serial of blockade assays, blockade of TGF-β signaling using the TβRIIDN or neutralizing antibody 1D11, decreased the levels of DNMTs between 50%–90% in more invasive cell lines, and to a lesser degree (30–50%) in the less invasive cell lines. These findings indicate that tumor-derived TGF-β is a major mediator involved in the regulation of DNMTs and TβRs in human CaP cells, and this process correlates with more invasive phenotypes. Meanwhile, down regulation of DNMT expression by blockade of TGF-β is associated with an up-regulation of naïve TβRs expression. These findings, taken together with results from our previous study, suggest that tumor-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1D11 treatment
13C4 treatment
No treatment
Day 14 after TRAMPC2 Challenge
Day 29 5 time anitbody injection
Day 44 10 time anitbody injection
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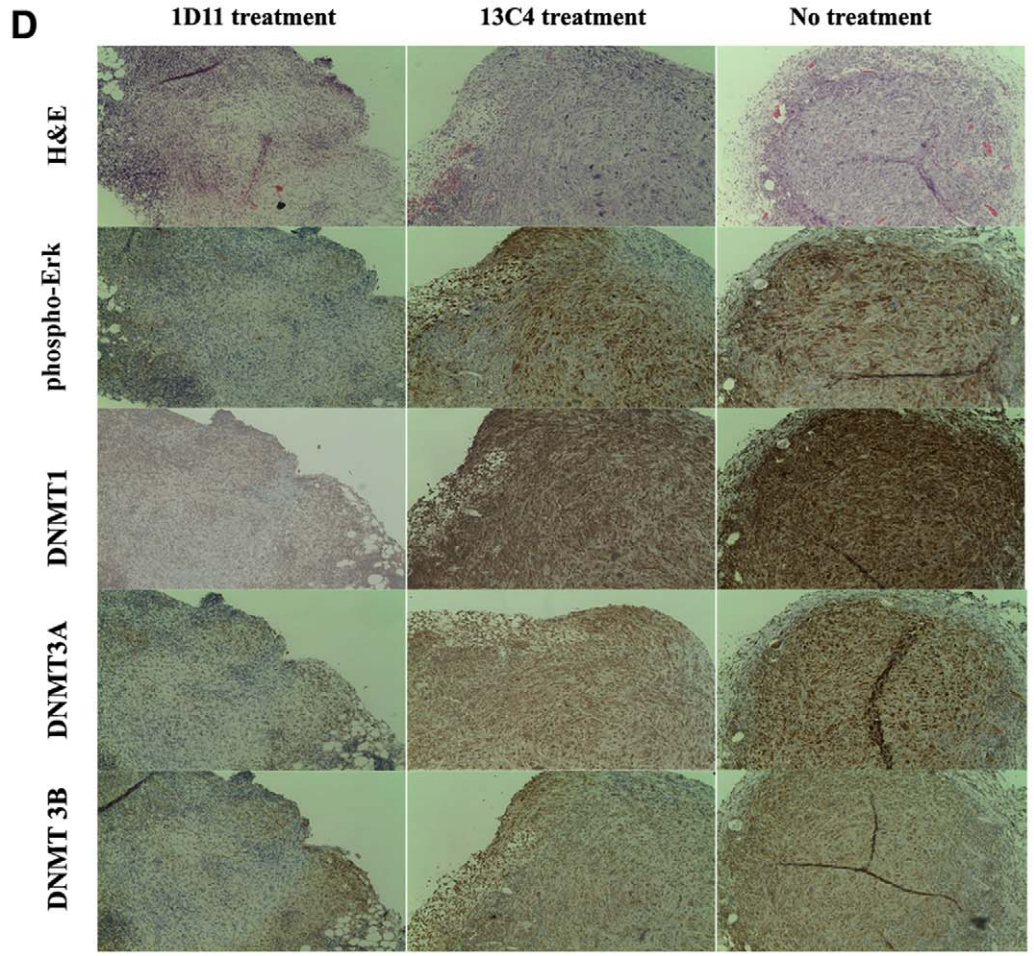
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Figure 4. TGF-β induced DNMTs is associated with growth of prostate cancer in vivo. A. IVIS 100 imaging system was used to monitor tumor growth in real-time. We found that tumor growth is inhibited dramatically with the treatment of 1D11 compared with Group 2 (13C4 treatment) and 3 (No treatment control). **B.** 1D11 treatment inhibits the tumor growth in a time dependent manner. **C,** At the end of the 45-day treatment period, mice were sacrificed and tumors were isolated. The average tumor weight and volume was 5.3 g and 6.85 cm^3, respectively in 1D11 treatment group. In comparison, the average weight and volume of tumors in the 10 animals treated with the control 13C4 was significantly greater at 15.8 g and 12.85 cm^3, respectively. The corresponding values in the mice that received no treatment were 31.4 g and 23.39 cm^3, respectively ($P<0.01$ among three groups). **D.** Immunohistochemical analyses of the primary tumors revealed that the expression of p-ERK, DNMTs in animals with 1D11 treatment is significantly lower than those of the other two groups.
doi:10.1371/journal.pone.0025168.g004

derived TGF-β activates ERK, which mediates the expression of DNMTs (because blockade of ERK resulted in 50% decrease on DNMTs expression). DNMTs then methylate the TGF-β receptor promoter regions resulting in the loss of growth inhibition mechanisms which we reported earlier [4]. Our present study also provides insight into the interaction between ERK and DNMTs in CaP. Exposure to the ERK inhibitor, UO126 results in >50% reduction in the expression of DNMTs, indicating that ERK is one of the major regulators of TGF-β induced DNMTs expression in CaP cells. Our observations of the co-localization of p-ERK and DNMTs also suggest that only cells which exhibit ERK activation can express DNMTs, which is evidence that they are in the same TGF-β activated signal pathway.

Importantly, we found direct evidence that blockade of DNMT by its inhibitor 5-Aza resulted in decrease in the invasive capabilities of CaP, as well as the blockade of either TGF-β by 1D11, or blockade of p-ERK by UO126. This data indicates that DNMT is a major promoter for CaP invasive capabilities. This procedure is regulated by TGF-β and mediated by p-ERK.

Based upon the above findings, we postulate that tumor-derived TGF-β can regulate its receptors by a potential feedback loop which is mediated by activation of ERK. Some other signaling factors like Serine/threonine protein phosphatases 2 (PP2A) [48] may be involved in this procedure. P-ERK may subsequently activate the transcription factors in the DNMTs promoter and increases the expression of DNMTs which methylates TGF-β receptor promoter regions resulting in the loss of growth inhibition mechanisms that are normally induced by TGF-β. Simultaneously, the downregulation of TβR expression and low level of TGF-β signaling may act as a positive feedback mechanism to induce the reflexive stimulation of TGF-β secretion in CaP. These potential feedback loops could explain the reduced expression of TβRs and large amounts of TGF-β that have been observed in advanced CaP. Our in vivo xenograft model also demonstrated that inhibition of DNMTs correlated to a lower tumor weight and cancer proliferation rate. These results suggest that the expression of DNMTs is associated with aggressive malignant phenotypes, tumor growth, and progression in vivo. In combination with our

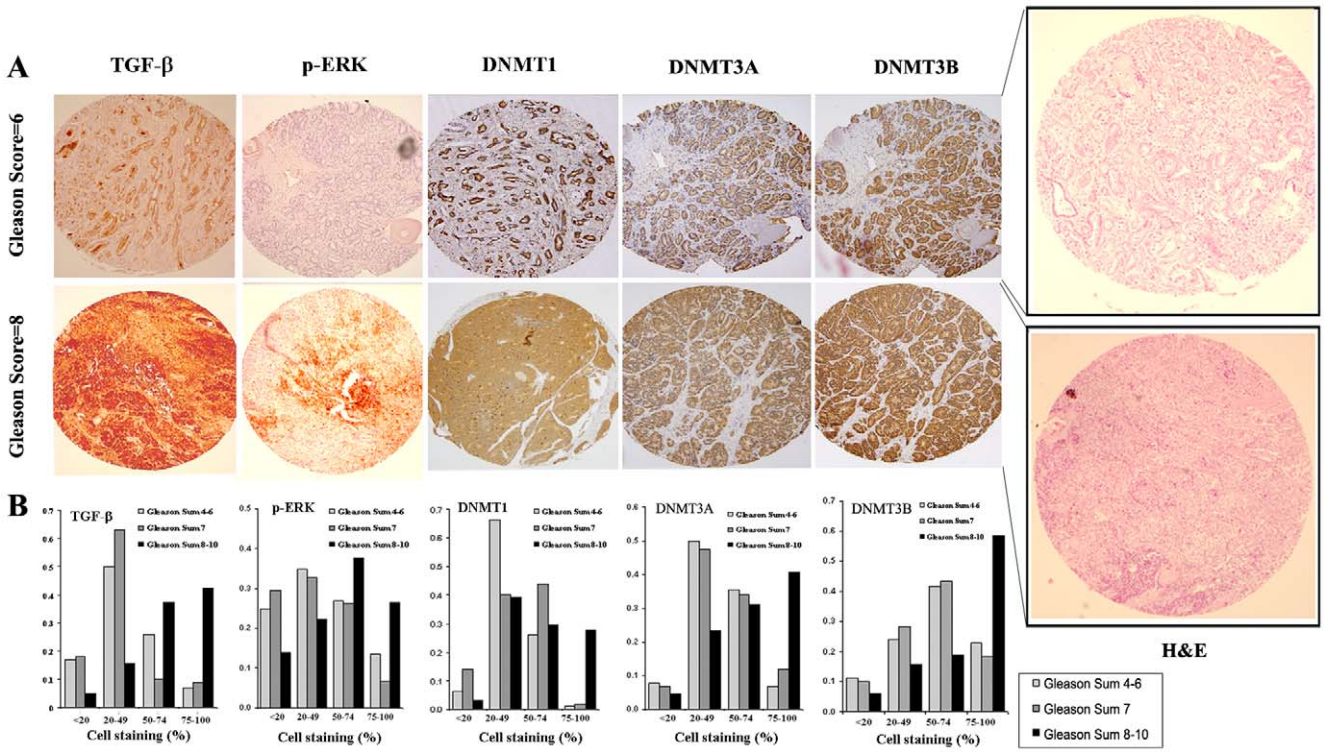

Figure 5. Confirmation of p-ERK mediated, TGF-β-induced DNMT in TMA specimens obtained at the time of prostatectomy. A. Immunohistochemical analysis of serial TMA sections from a patient with Gleason's score of 8, revealed higher expression of TGF-β, p-ERK, DNMTs, but lower expression of TβRI and TβRII and p-Smad2 as compared to serial sections taken from a patient with a lower Gleason's grade of 6. These results are representative of the predominant staining pattern seen in all patient samples/tissue arrays (magnification: 10×20). The corresponding frequency (or percentage) of staining and intensity of staining could be found on B. **B.** High levels of TGF-β1 expression (Score = 3) were identified in 42.3%, 8.9% and 7.0%, p-Erk expression in 36.4%, 6.7% and 13.5%, DNMT1 expression in 27.9%, 1.8% and 1.3%, DNMT3A expression in 40.6%, 11.9% and 6.7%, and DNMT3B expression in 58.7%, 18.3% and 22.8% of high (≥8), intermediate (= 7), and low Gleason grade (≤6), respectively.
doi:10.1371/journal.pone.0025168.g005

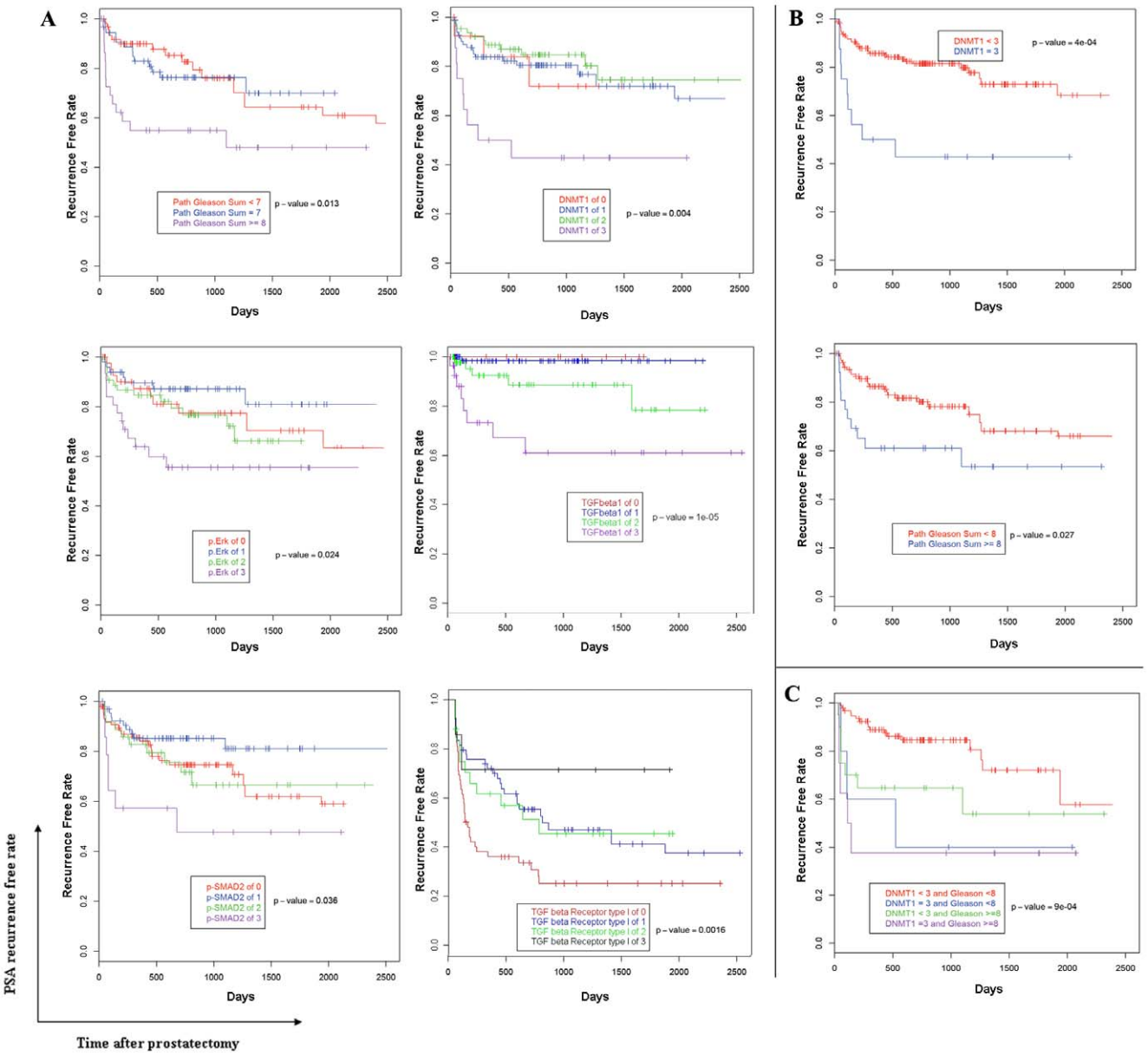

Figure 6. TGF-β-induced DNMT1 predicts prostate cancer recurrence. A. Kaplan Meier curve was generated for significant variables. TGF-β1, p-Smad2, p-ERK, pathologic Gleason Score and DNMT1, TβRI were all predictors of biochemical recurrence. **B.** DNMT1, pathologic Gleason score was analyzed as two different groups, and there was a significant difference in the survival curves. **C.** Cox Proportional Hazards Model only includes DNMT1, grouped as below 3 and 3, and pathologic Gleason score sum of patients, grouped as below 8, or above. Using our dataset, patients with tissue level DNMT1 of 3 had a 3.53 times higher biochemical recurrence rate than patients with lower tissue levels of DNMT1. Patients with Gleason score sum $\geq$8 have a 2.27 times higher biochemical recurrence rate compared to patients with Gleason score sum $<$8.
doi:10.1371/journal.pone.0025168.g006

previous findings [4], we found that DNMTs is an important factor and predictor related to CaP progression.

Furthermore, the close correlation between TGF-β, ERK and DNMTs in tissue microarray specimens indicates that this cascade of signal events is likely not only associated with aggressive malignant phenotypes in vitro, but may also be involved with progression of CaP in humans. Based on our results, during progression of prostate cancer, an attenuation of expression of TGF-β receptors facilitates tumor cells escaping from the growth inhibition by TGF-β which is Smad dependent. Meanwhile, the Smad-independent pathway, such as p-ERK and DNMT signaling could be induced by TGF-β and results in the more aggressive phenotypes.

Our data shows that increased expression of DNMTs is highly correlated with both the expression levels of TGF-β1 and p-ERK. Furthermore, there was a significant correlation between the levels of DNMTs and Gleason grade, which further supports our findings that DNMTs are associated with more invasive CaP phenotypes. This finding is similar to recent reports suggesting that DNMT1 is associated with lung cancer progression [49]. The present results demonstrate that DNMT1 is associated with biochemical recurrence in CaP patients seven years following radical prostatectomy. Thus, patients with higher tissue expression levels of DNMT1 are at increased risk for biochemical recurrence compared to those with lower tissue expression levels. The relationship between DNMT1

expression and biochemical recurrence is independent of Gleason grade. Although other variables including TGF-β1, p-ERK, Gleason grade were also showed significantly associated with biochemical recurrence, the final Cox Proportional Hazards Model demonstrated that DNMT1, in combination with pathologic Gleason sum, are stronger predictors for disease outcome. The exact mechanism of this observation remains unclear, but variables involved in the signal pathway including tumor expression of DNMT1, TGF-β1, and p-ERK may be useful in predicting clinical outcome following radical prostatectomy. High expression level of DNMT1 was risk factors for biochemical recurrence in men with CaP, regardless of Gleason's score.

In summary, our findings indicate that DNMTs expression levels are correlated with invasive capabilities in cultured human CaP cell lines. Additionally, we found that tumor-derived TGF-β and ERK are involved in the regulation of DNMTs in these cell lines. Inhibition of TGF-β in vivo results in the corresponding inhibition of DNMTs, and appears to significantly decrease tumor growth. In addition, we confirmed that the expression levels of TGF-β, ERK and DNMTs in tissue specimens obtained at the time of prostatectomy mimicked our findings in cell culture. Finally, we found that high expression levels of DNMT1 may potentially be used to predict biochemical recurrence in patients following radical prostatectomy.

Supporting Information

Figure S1 Immunoblot analyses demonstrated that after treatment with 5-Aza-2′-deoxycytidine (5-Aza), the expression of TβRI and TβRII in PC-3 increased dramatically. In contrast, the expression of both TβRI and TβRII decreased significantly with the treatment of TGF-β and this change could be recovered when 5-Aza is added (Figure S1A). Similarly, real-time PCR confirmed that the expression of both TβRI and TβRII was increased 2–2.5 folds after treatment of 5-Aza in PC-3 cells. Treatment of TGF-β suppressed the expressions of TβRI and TβRII 46% and 29% respectively (Figure S1B). We also identified the methylation status of TβRI and TβRII promoters, by using the same MSP approach and sequencing methodologies (4). Using this technique, we found the same methylated sites as our previous study (4) in that cytosine positions −251, −231, −244, −348, −356 and −365 in the promoter of TβRI, and +27, +32 and −140 for the promoter of TβRII were methylated (Figure S1C). PC-3 cells also have a portion of TβRI and TβRII promoters that are unmethylated. Interestingly, treatment with TGF-β increased the methylation status, but treatment with 5-Aza converted all methylated sites to unmethylated. The thymidine incorporation assay indicated that the proliferation of PC-3 cells were only modestly inhibited modestly by exogenous TGF-β. In comparison, 5-Aza treatment resulted in a significant inhibition of cell proliferation, regardless of whether exogenous TGF-β was added into the culture or not. There was no significant difference observed between treatment with both 5-Aza and TGF-β or with 5-Aza alone ($P>0.05$) (Figure S1D). Taken together, these results support our above finding that knockdown of DNA methyltransferases result in the demethylation of the TGF-β receptors gene promoters and restoration of TGF-beta inhibition of cell growth. TGF-β contributes to the methylation of its own receptors. **A.** Immunoblot analyses demonstrated that after treatment with 5-Aza-2′-deoxycytidine (5-Aza), the expression of TGF-β receptor I (TβRI) and TGF-β receptor II (TβRII) in PC-3 increased dramatically. In contrast, the expression of both TβRI and TβRII decreased significantly with the treatment of TGF-β and this change could be recovered when 5-Aza is added. **B.** Real-time PCR confirmed that the expression of both TβRI and TβRII was increased 2–2.5 folds after treatment of 5-Aza in PC-3 cells. Treatment of TGF-β suppressed the expressions of TβRI and TβRII 46% and 29% respectively. **C.** The methylation status of TβRI and TβRII promoters was identified by using the same MSP approach and sequencing methodologies (4). The methylated sites in cytosine positions −251, −231, −244, −348, −356 and −365 in the promoter of TβRI, and +27, +32 and −140 for the promoter of TβRII. PC-3 also has portion of TβRI and TβRII promoters which are unmethylated. Interestingly, treatment with TGF-β also increased the methylation status, but treatment with 5-Aza can convert all methylated sites to unmethylated. **D.** The thymidine incorporation assay indicated that the proliferation of PC-3 could be inhibited little by exogenous TGF-β. However, 5-Aza treatment resulted in a significant inhibition of cell proliferation, regardless of whether exogenous TGF-β was added into the culture or not. There was no significant difference observed between treatment with both 5-Aza and TGF-β or with 5-Aza alone ($P>0.05$).
(TIF)

Figure S2 For positive control staining for TMA staining, a tissue (Colon cancer for TGF-β, Breast cancer for p-ERK, Placenta for DNMT1 and DNMT3A, Breast cancer for DNMT3B respectively) which is well known to have expression of target protein was used. Negative controls were identical array sections stained in the absence of primary antibody.
(TIF)

Method S1 Supplemental Materials and Methods.
(DOC)

Method S2 NU Pathology Core Facility Standard Operating Procedure.
(DOC)

Table S1 Secretion of TGF-β in various derivatives of PC3 cells and benign cells (pg/ml/48 hours/10^5 cells).
(DOC)

Table S2 Clinical and pathologic characteristics of patients who experienced a radical prostatectomy.
(DOC)

Table S3 Correlation of TGF-β signaling components and clinical characterics.
(DOC)

Table S4 The variables significantly correlated with biochemical recurrence.
(DOC)

Table S5 The significant variables selected by Cox Proportional Hazards Mode.
(DOC)

Letter S1 Northwestern University ACUC Approval protocol number 2007-0565.
(PDF)

Letter S2 Approval letter by the Northwestern University Institutional Review Board (The IRB number is 1480-002).
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: QZ LC BH CL. Performed the experiments: QZ LC BH CL. Analyzed the data: QZ LC BH CL TJ JK BJ XY NY YG TK CB SL JH BT WC LZ VS. Contributed reagents/materials/analysis tools: QZ LC BH CL TJ JK BJ XY NY YG TK CB SL JH BT WC LZ VS. Wrote the paper: QZ LC CL BH TJ.

References

1. Sintich SM, Lamm MLG, Sensibar Jam Lee C (1999) Transforming growth factor-ß1 induced proliferation of the prostate cancer cell line, TSU-Pr1: the role of platelet-derived growth factor. Endocrinology 140: 3411–3415.
2. Zhou W, Park I, Pins M, Kozlowski JM, Jovanovic B, et al. (2003) Dual regulation of proliferation and growth arrest in prostatic stromal cells by transforming growth factor-ß1. Endocrinology 144: 4280–4284.
3. Pardali K, Moustakas A (2007) Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta 1775: 21–62.
4. Zhang Q, Rubenstein JN, Jang TL, Pins M, Javonovic B, et al. (2005) Insensitivity to transforming growth factor-beta results from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). Mol Endocrinol 19: 2390–2399.
5. Robertson KD (2001) DNA methylation, methyltransferases and cancer. Oncogene 20: 3139–3155.
6. Bestor TH (2000) DNA-methyltransferase of mammals. Hum Mol Genet 9: 2395–2402.
7. Okano M, Bell DW, Haber DA, Li E (1999) Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99: 247–257.
8. Lu R, Wang X, Chen ZF, Sun DF, Tian XQ, et al. (2007) Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells. J Biol Chem 282: 12249–12259.
9. Saito Y, Yamazoe T, Qin Z, Ohgomori K, Mochitate K, et al. (2003) Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 105: 527–532.
10. Etoh T, Kanai Y, Ushijima S, Nakagawa T, Nakanishi Y, et al. (2004) Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 164: 689–699.
11. Xing J, Stewart DJ, Gu J, Lu C, Spitz MR, et al. (2008) Expression of methylation-related genes is associated with overall survival in patients with non-small cell lung cancer. Br J Cancer 98: 1716–22.
12. Amara K, Ziadi S, Hachana M, Soltani N, Korbi S, et al. (2010) DNA methyltransferase DNMT3b protein overexpression as a prognostic factor in patients with diffuse large B-cell lymphomas. Cancer Sci 101: 1722–1730.
13. Chen MF, Chen WC, Chang YJ, Wu CF, Wu CT (2010) Role of DNA methyltransferase 1 in hormone-resistant prostate cancer. J Mol Med 88: 953–962.
14. Luo X, Zhang Q, Liu V, Xia Z, Pothoven KL, et al. (2008) Cutting edge: TGF-beta-induced expression of Foxp3 in T cells is mediated through inactivation of ERK. J Immunol 180: 2757–2761.
15. Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Lang S, et al. (1996) Loss of expression of transforming growth factor-β receptors type I and type II correlates with tumor grade in human prostate cancer tissues. Clinical Cancer Research 2: 1255–1261.
16. Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Sensibar JA, et al. (1996) Genetic change in transforming growth factor-β (TGF-β) receptor type I gene correlates with insensitivity to TGF-β1 in human prostate cancer cells. Can Res 56: 44–48.
17. Pettaway CA, Pathak S, Greene G, Ramirez E, Wilson MR, et al. (1996) Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Caner Res 2: 1627–1636.
18. Kozlowski JM (1988) Prostate cancer and the invasive phenotype: application of new in vivo and in vitro approaches. In: Fidler IJ, Nicholson G, eds. Tumor progression and metastasis. New York: Alan R Liss Inc. pp 189–231.
19. Kozlowski JM (1991) Growth requirements of human prostate cancers in vitro and in vivo. In: Rosen S, Lee C, Tallman MS, eds. Innovations in Urologic Oncology: Selected Papers from a Northwestern University Cancer Center Symposium. Chicago: Precept Press. pp 13–37.
20. Lee C, Shevrin DH, Kozlowski JM (1993) In vivo and in vitro approaches to study metastasis in human prostatic cancer. Cancer Met Rev 12: 21–28.
21. Zhang Q, Yang X, Pins M, Javonovic B, Kuzel T, et al. (2005) Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive $CD8^+$ T cells: eradication of autologous mouse prostate cancer. Cancer Res 65: 1761–1769.
22. Zhang Q, Helfand BT, Jang TL, Zhu LJ, Chen L, et al. (2009) Nuclear factor-kappaB-mediated transforming growth factor-beta-induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy. Clin Can Res 15: 3557–3567.
23. Liu VC, Wong LY, Jang T, Shah AH, Park I, et al. (2007) Tumor evasion of the immune system by converting CD4+CD25- T cells into $CD4^+CD25^+$ T regulatory cells: role of tumor-derived TGF-beta. J Immunol 178: 2883–2892.
24. Perry K, Wong L, Liu V, Park I, Zhang Q, et al. (2008) Treatment of transforming growth factor-beta-insensitive mouse Renca tumor by transforming growth factor-beta elimination. Urology 72: 225–229.
25. Zhang Q, Yang XJ, Kundu SD, Pins M, Javonovic B, et al. (2006) Blockade of transforming growth factor-{beta} signaling in tumor-reactive CD8(+) T cells activates the antitumor immune response cycle. Mol Cancer Ther 5: 1733–1743.
26. Attwood J, Richardson B (2004) Relative quantitation of DNA methyltransferase mRNA by real-time RT-PCR assay. Methods Mol Biol 287: 273–283.
27. Serganova I, Moroz E, Vider J, Gogiberidze G, Moroz M, et al. (2009) Multimodality imaging of TGFbeta signaling in breast cancer metastases. FASEB J 23: 2662–2672.
28. Ponomarev V, Doubrovin M, Serganova I, Vider J, Shavrin A, et al. (2004) Novel triple-modality reporter gene for whole-body fluorescent, bioluminescent, and nuclear noninvasive imaging. Eur J Nucl Med Mol Imaging 31: 740–751.
29. Steg A, Vickers SM, Eloubeidi M, Wang W, Eltoum IA, et al. (2007) Hedgehog pathway expression in heterogeneous pancreatic adenocarcinoma: implications for the molecular analysis of clinically available biopsies. Diagn Mol Pathol 16: 229–237.
30. Grizzle WE, Myers RB, Manne U, Srivastava S (1998) Immunohistochemical Evaluation of biomarkers in prostate and colorectal neoplasia: Principles and guidelines. In: Methods in Molecular Medicine, Vol 14: Tumor Marker Protocols Hanausek M, Walaszek Z, eds. Humana Press, Inc, Totowa, NJ. pp 143–160.
31. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP (1998) Alterations in DNA methylation: A fundamental aspect of neoplasia. Adv Cancer Res 72: 141–196.
32. Chan MF, Liang G, Jones PA (2000) Relationship between transcription and DNA methylation. In: Jones PA, Vogt PK, eds. DNA methylation and cancer. New York: Springer-Verlag. pp 75–99.
33. Bender CM, Pao MM, Jones PA (1998) Inhibition of DNA methylation by 5-AZA-2′ deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 58: 95–101.
34. Santi DV, Garrett CE, Barr PJ (1983) On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. Cell 33: 9–10.
35. Wu JC, Santi DV (1987) Kinetic and catalytic mechanism of HhaI methyltransferase. J Biol Chem 262: 4778–4786.
36. Bacolla A, Pradhan S, Roberts RJ, Wells RD (1999) Recombinant human DNA (cytosine-5) methyltransferase II Steady-state kinetics reveal allosteric activation by methylated DNA. J Biol Chem 274: 33011–33019.
37. Deng C, Lu Q, Zhang Z, Rao T, Attwood J, et al. (2003) Hydralazine may induce autoimmunity by inhibiting extracellular signal-regulated kinase pathway signaling. Arthritis Rheum 48: 746–756.
38. Lu Q, Wu A, Richardson BC (2005) Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. J Immunol 174: 6212–6219.
39. Yu N, Wang M (2008) Anticancer drug discovery targeting DNA hypermethylation. Curr Med Chem 15: 1350–1375.
40. Ghoshal K, Bai S (2007) DNA methyltransferases as targets for cancer therapy. Drugs Today (Barc) 43: 395–422.
41. Gowher H, Jeltsch A (2004) Mechanism of inhibition of DNA methyltransferases by cytidine analogs in cancer therapy. Cancer Biol Ther 3: 1062–1068.
42. Fandy TE (2009) Development of DNA methyltransferase inhibitors for the treatment of neoplastic diseases. Curr Med Chem 16: 2075–2085.
43. Patra SK, Patra A, Zhao H, Dahiya R (2002) DNA methyltransferase and demethylase in human prostate cancer. Mol Carcinog 33: 163–171.
44. MacLeod AR, Rouleau J, Szyf M (1995) Regulation of DNA methylation by the Ras signaling pathway. J Biol Chem 270: 11327–11337.
45. Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, et al. (2007) TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J 26: 3957–67.
46. Giehl K, Seidel B, Gierschik P, Adler G, Menke A (2000) TGF beta1 represses proliferation of pancreatic carcinoma cells which correlates with Smad4-independent inhibition of ERK activation. Oncogene 19: 4531–4541.
47. Lin C, Zhang Q, Helfand B, Qin W, Sharma V, et al. (2010) Erk Activation mediates Transforming Growth Factor-ß-induced up-regulation of DNA methyltransferase in human prostate cancer cells. 101th, American Urological Association Annual Meeting San Francisco, CA AB-1001488.
48. Zhang Q, Chen L, Helfand B, Kozlowski J, Brendler C, et al. (2011) The recruitment of PP2A by TGF-β receptors mediates the response to TGF-β-induced activation of ERK in prostate cancer. 2011 102^{nd} American Association of Cancer Research (AACR) Annual meeting, Orlando, FL. Cancer Research, Late-breaking abstract LB-3.
49. Lin RK, Hsieh YS, Lin P, Hsu HS, Chen CY, et al. (2010) The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients. J Clin Invest 120: 521–532.

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The recruitment of PP2A by TGF-β receptors mediates the response to TGF-β-induced activation of ERK in prostate cancer.

Short Title:
PP2A regulates p-ERK level in CaP

Author Block *Qiang Zhang*, *Lin Chen*, *Brian Helfand*, *James Kozlowski*, *Charles B Brendler*, *Timothy M. Kuzel*, *Chung Lee*. Northwestern Univ., Chicago, IL, NorthShore University, Chicago, IL

Abstract:
Introduction and Objective: We recently demonstrated that TGF-β mediated phosphorylation of extra-cellular signal-regulated kinase (ERK) activation results in the prostate cancer (CaP) progression and metastasis. Serine/threonine protein phosphatases 2, PP2A (including subunit -A, -B and -C) are well known to be involved in the dephosphorylation and inactivation of ERK. In this study, we determined the association between the recruitment of PP2A by TGF-β receptors (TβRI and TβRII) and activation of ERK under the treatment of TGF-β.
Methods: The human CaP cell lines PC-3 with different capability of aggressive (PC-3, PC-3M, PC-3M-Pro and PC-3M-LN4), and benign prostate epithelial cell line BPH-1 were used for these studies. Cells were treated with TGF-β (10ng/mL) for 24 minutes. The expression of phospho-ERK (p-ERK), total-ERK (t-ERK), TβRs was evaluated by quantitative western blot analyses. The conjugation of PP2A (-A, -B and -C) and TβRI and TβRII were elucidated using western blot following immunoprecipitation (IP, Pierce Crosslink kit) with TβRI and TβRII as the precipitant respectively. Briefly, precleared lysate was immunopreciptated by the crosslinked TβRI or TβRII antibody (5 μg) and agarose mixture for overnight on 4°C. Control agarose resin in the kit was used as a negative control when western-blot for PP2A was conducted. The recruitment of PP2A by TβRs was correlated with the expression of p-ERK and TβRs.

Results: TGF-ß treatment resulted in an increase in p-Erk expression (4-fold) in all PC-3 cell lines in a time dependent manner post TGF-ß exposure. In addition, the expressions of TßRI and TßRII were suppressed by 46% and 29% respectively. IP studies revealed recruitment of PP2A conjugated with TßRI and TßRII. In contrast, the expression of p-Erk was dramatically inhibited in BPH-1 by TGF-ß exposure. Although there is no significant change on the expression of TßRs, the ratio of PP2A versus TßRII was significantly increased from 2.08 to 3.12, which suggests that the recruitment of PP2A was relatively increased in BPH-1 cells under the treatment of TGF-ß. Finally, there was a reverse correlation between recruited PP2A and activation of p-ERK in both PC-3 cell lines, and BPH-1 cells.
Conclusion: Taken together the results suggest that TGF-β suppresses the recruitment

of PP2A by TGF-β receptors in CaP cells in contrast to benign prostate cells, which results in relatively increased activation of ERK and the subsequent tumor progression. The identification of the recruitment of PP2A by TβRs represents a new focus to elucidate in part how TGF-β plays a different, or even a contrary role in CaP and benign cell respectively. PP2A may be a potential new target for CaP therapies.

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Anti-transforming growth factor beta antibody 1D11 suppresses the invasion of human prostate cancer cells

Introduction and Objective: Metastases are responsible for disease specific mortality in men with prostate cancer (CaP). Previously, we reported that TGF-β induced vimentin expression is associated with the epithelial-to-mesenchymal transition (EMT) which is correlated with migration of CaP cells and a worse prognosis in clinical specimens obtained at prostatectomy. In this study, we explored whether a specific neutralizing anti-TGF-β antibody 1D11 inhibits the migratory and invasive potential of CaP cells.

Methods: Cell invasion assays were performed using the human CaP cell lines DU145 and PC-3 in a 24-well matrigel transwell chamber (8-μm pore size; CytoSelect^{TM}; Cell Biolabs). Four different treatment groups were assigned to each cell line: Group 1 - no treatment (control); Group 2 - treatment with TGF-β (10ng/mL) for 24 hours; Group 3 - treatment with anti-TGF-β (1, -2, -3) neutralizing mAb clone 1D11 (5ug/mL) for 24 hours; and Group 4 – treatment with ERK inhibitor U0126 5μM. After 24 hours of invasion, the invaded cells were counted with a light microscope under high magnification objective (100×) and were then lysed and measured at OD 560nm in a plate reader after treatment with the extraction solution. Furthermore, Western blot analyses were performed to evaluate the expression of vimentin in each treatment group.

Results: An average of 82 cells/field were found to invade in the control group. In Group 2, there was a significant increase in the number of invaded cells (139.33/field) with TGFB treatment. Invasion of PC3 cells was significantly inhibited by treatment of 1D11, (only 7.67 cells/field). The ERK inhibitor, U0126, was also associated with a significant decrease in the number of invasive cells (14.67 cells/field; Group 4). Consistent with the results of the migration assay, Western blot analyses showed that after treatment with TGF-β, the expression of vimentin increased by 2-2.5 folds. In contrast, with the treatment of 1D11 and U0126, the expression of vimentin was suppressed 70% and 40% respectively. Furthermore, treatment of 1D11 also resulted in the 50% inhibition of p-ERK, which indicated that 1D11 may inhibit TGF-β induced vimentin through a p-ERK pathway.

Conclusion: We previously reported TGF-β induced EMT is associated with more aggressive CaP phenotypes. The present findings indicate that neutralization of tumor secreted TGF-β by 1D11 may inhibit TGF-β and ERK induced expression of vimentin. Phenotypically this results in the suppression of CaP migration. Our results suggest potential targets for the future development of effective anti-tumor therapeutic strategies.

Slide One: Metastasis is responsible for disease specific mortality in prostate cancer patients. Previously, we reported that TGF-β can induce epithelial-to-mesenchymal transition (EMT) which is correlated with migration of CaP cells and a worse prognosis in clinic. In this study, we explored whether a neutralizing anti-TGF-β antibody 1D11 inhibits the migratory and proliferation CaP cells.

Slide Two: Metragel migratiion assays showed in control group, an average of 99 cells/field were found to invade the metragel. Treatment of TGF-B dramatically increase the invaded cells. But, invasion of prostate cancer cells was significantly inhibited by treatment of 1D11, or the ERK inhibitor, U0126.

In spite of the metragel assay, we reported before, treatment of 1D11 suppress the expression of P-erk and vimentin was suppressed by 50%, which indicated that 1D11 may inhibit TGF-β induced vimentin and migration through a p-ERK pathway.

Slide Three: In the in vivo study, we established mice prostate cancer model by injection of TRAMP-C2 cells. Then mice received I.P injection of 1D11 or a control antibody 13C4, every three days. We follow up the tumor growth by luciferase assay.. We found, 1D11 can significantly suppress the tumor growth in vivo.

Conclusion: Taken together, our in vitro and in vivo studies indicate that neutralization of tumor secreted TGF-β by 1D11 may inhibit TGF-β induced tumor migration or proliferation. ID11 is a potential target for the future development of anti prostate cancer therapeutic strategies.

Title:
Natural products and transforming growth factor-beta (TGF-β) signaling in cancer development and progression

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ABSTRACT:

TGF-β can inhibit growth of benign cells but promote metastasis in advanced cancer. Actions of many herbal medicine products for cancer treatment are linked to an altered production of TGF-β in the target cells. The effect of herbal natural products on cancer can be divided into three categories. The first category of herbal medicine products will be those related to the induction TGF-β in target cells. Examples in this category include seaweed, resveratrol, green tea, curcumin, etc. Any herbal medication in this category will be able to prevent cancer. However, they may not necessarily be beneficial to patients with established cancer. The second category of herbal products will inhibit TGF-β signaling and inhibit TGF-β mediated growth promotion and metastasis in advanced cancers. Examples in the second category include genistein, rhubarb, Long Dan Tan, etc. Finally, the third category of herbal products have no impact on TGF-β signaling, such as lycopene.

Keywords:

Dietary components, TGF-β signaling, DNA methylation. Tumor development and progression, Smad pathways, non-Smad pathways.

INTRODUCTION:

Many herbal products used for cancer prevention and treatment have an effect on TGF-β signaling, it is therefore important that we understand the action of these herbal products in relationship to TGF-β signaling. It is known that TGF-β can inhibit growth in benign cells and in early stage cancer cells, but it will promote progression and metastasis in advance stage cancers. With this differential property of TGF-β between benign and cancer cells, it is important that we must select the right type of herbal products for cancer prevention and other type of herbal medication for cancer treatment. In this review, we will briefly discuss three categories of herbal products used in prevention and treatment of cancer patients. The first class of herbal products will induce TGF-β production in the target cells. The second class of herbal products will inhibit TGF-β signaling. The third class of herbal products has no effect on TGF-β signaling.

Biology of TGF-β signaling and TGF-β paradox:

TGF-β was first described in the early 1980s [1-3]. It represents a family of pleiotropic growth factors with diverse functions, such as embryonic development, wound healing, organ development, immuno-modulation, and cancer progression [4, 5]. There are three known mammalian isoforms of TGF-β (TGF-β1, -β2, and -β3) with significant homology and similarities in function. The biological effect of TGF-β is mediated through type I, type II receptors and downstream transcription factors, Smad [6, 7]. While this is an accepted pathway for TGF-β signaling, other non-Smad signaling pathways, which lack the classical growth inhibitory functions of TGF-β, have also been identified, such as MAPK, PI3K/AKT, PP2A/p70s6K, and JNK [8-10]. The relative importance and interplay of these various pathways of TGF-β signaling are still under investigation [11-14]. In general, events mediated through the Smad pathways are mainly related to growth arrest and apoptosis; while those mediated through the non-Smad pathways are mainly related to cell proliferation and migration [8].

The well known TGF-β paradox is that TGF-β is a potent inhibitor to benign cells but promotes proliferation and invasion in cancer cells [15]. The molecular mechanism of this TGF-β paradox remains unexplained. The inhibition of cell proliferation by TGF-β is due to cell cycle arrest in the G1 phase. The signaling system responsible for this growth arrest mechanism includes activation of TGF-β receptors (type I and type II) and their downstream transcription factors, Smad signal transducers. However, in cancer cells, the inhibitory property of TGF-β is greatly diminished. In the majority of advanced cancer cases, TGF-β enhances proliferation, invasion, metastasis and evasion of host immune surveillance. In addition to the loss of responsiveness of the cancer cells to TGF-β mediated growth inhibition, these cells secrete increasing amounts of TGF-β, which itself serves as a pro-malignant factor by suppressing anti-tumor immune response of the host and by augmenting angiogenesis [16-18]. **Figure 1** depicts a simplified TGF-β signaling in relation to activation of Smad2/3 and Erk1/2 in benign and cancer cells.

(Please place **Figure 1** here)

Differential activation of Erk between benign and cancer cells:

The effect of TGF-β on proliferation varies according to the type of target cells. In our recent study [18], we observed a differential regulation of proliferation and growth arrest between normal (benign) and cancer cells in response to TGF-β. We have investigated the mechanism of this dual effect on proliferation and growth arrest by TGF-β. Although TGF-β mediates Erk activation at low doses (0.1 ng/ml) in both benign and cancer cells, at high doses (10 ng/ml), TGF-β treatment resulted in an inactivation of Erk in benign cells but continue to activate Erk in cancer cells [18]. This differential activation of Erk in cancer cells by TGF-β but not in benign cells has not been appreciated

before and provides the answer to the known TGF-β paradox. Since activated Erk is a master regulator of tumor progression, it mediates a series of downstream events, such as NF-κB activation, up-regulation of DNMT, down-regulation of E-cadherin, enhanced action of β-catenin/Wnt, expression of vimentin, and induction of Smad7, that perpetuate a vicious cycle in TGF-β signaling leading to a continuous cancer growth and progression [19-24].

A candidate protein responsible for this differentially regulated TGF-β signaling between benign and malignant cells is protein phosphatase 2A (PP2A). PP2A is ubiquitously synthesized in the cell cytoplasm but is recruited by the activated TβRI to the cell membrane to function as an inhibitor to Erk activation [18, 25-27]. In the benign cells, PP2A will be recruited by the activated TβRI, which is dependent on the dosage of TGF-β available to the target cells. At a high dose of TGF-β, a sufficient quantity of PP2A is recruited by the activated TβRI, resulting in inhibition of Erk activation; while at a low dose of TGF-β, due to a limited quantity of TβRI being activated, there will be a limited quantity of PP2A available resulting in Erk activation. In the context of malignant cells, due to a severely down-regulated TβRI, recruitment of PP2A is always at a limited level regardless of the dosage of TGF-β employed in the environment, resulting in a constant activation of Erk. This differential Erk activation between benign and malignant cells offers an explanation of the vicious cycle of TGF-β signaling leading to a continuous tumor growth and progression.

TGF-β mediated vicious cycle in tumor progression:

Activated Erk is responsible for a host of signaling events that lead to tumor progression. Activated Erk will mediate loss of E-cadherin and over-expression of vimentin, a hallmark of epithelial-to-mesenchymal transition (EMT) in cancer cells. Additional downstream events of Erk activation include Smad7 expression [23], NF-κB activation [19] and β-catenin/Wnt signaling [28]. Most notable events are auto-induction of TGF-β expression and down-regulation of TβRs [18, 19], thus creating a unique tumor microenvironment that eventually lead to a vicious cycle in tumor progression. This TGF-β mediated vicious cycle in tumor progression has a multitude of detrimental physiological ramifications. These include DNA hypermethylation, proliferation/loss of the apoptotic response, epithelial-mesenchymal transition (EMT) and metastasis, evasion of immune system surveillance, and sustained angiogenesis [19, 29-31]. Effects of this unique TGF-β mediated microenvironment on each of these phenomena will be discussed in the following sections.

Crosstalk between adjacent stromal and epithelial cells – a TGF-β story:

During embryogenesis, epithelial cells in the ectoderm change into mesenchymal cells, which migrate through the primitive streak and insert themselves between the ectoderm and the endoderm. Some of these mesenchymal cells will engage in the establishment and maintenance of a lifelong relationship with the epithelial cells. The cross talk between two cellular components is mainly centered on the TGF-β signaling. TGF-β, under normal physiological conditions, is a gate keeper to maintain cellular homeostasis, including the maintenance of the normal structural and functional integrity of the organ. Any variation within this delicate balance between stromal and epithelial cells will result in pathological conditions, such as abnormal development, fibrosis, and even cancer. For example, an imbalance in TGF-β signaling by a reduction in TGF-β sensitivity and an increase in TGF-β production in the stromal cells is able to bring about malignant transformation in the adjacent epithelial cells [32, 33]. In cancer, both the cancer cells and the adjacent CAFs will send oncogenic signals the fuel the cancer progression and metastasis [34, 35]. Again, an aberrant TGF-β signaling in the both stromal and epithelial compartments sets off a vicious cycle leading to a continuous tumor progression and metastasis [36, 37]. Cancer cells can trans-differentiate the adjacent fibroblasts to myo-fibroblasts, which can lead to many activities of TGF-β mediated events in cancer. These events

include changes in cytokine balances, EMC proteins, proteases and their inhibitors, resulting in cancer invasion and ectopic survival, angiogenesis, and evasion of host immune surveillance program [37-39]. In addition to contributing to cytokines, modified ECM, proteases and protease inhibitors, myofibroblasts themselves are able to invade into cancer cell compartment [40].

Three classes of natural products in relation to TGF-β production in target cells:

In light of the above discussion, it is apparent that TGF-β signaling is an important biological event that can impact on cancer development and progression. Through an extensive literature survey, we found that herbal products can either stimulate TGF-β production or inhibit TGF-βsignaling, while others have no effect on TGF-β signaling. **Table 1** lists three classes of natural products in relation to TGF-β production. Class I illustrates some examples of natural products that induce TGF-β production in target cells. Class II are natural products that inhibit TGF-β signaling; while Class III are examples of natural products that have no relation to TGF-β production.

Herbal products that induce TGF-β production in target cells

Many herbal products have a protective effect against carcinogenesis. Aside from having the anti-oxidant property, they also induce TGF-β expression from the target cells. Since TGF-β is growth inhibitory and can induce apoptosis in normal non-cancer target cells, such property will be a suitable anti-cancer supplements for cancer prevention. Examples for these products include seaweed [41] and resveratrol [42]. Other products, such as Inchin-ko-to [43] and Long Dan Tan [44], also have the ability to induce TGF-β production in target cells. Additional studies revealed that Scutellaria baicalensis Georgi (Sb) and Bupleurum scorzonerifolfium Willd (Bs) can inhibit cell proliferation by an increase in TGF-β production in target cells [45]. Euonymus alatus (Thunb.) Sieb (EA), known as "gui-jun woo" in Korea, which is used for leiomyomal tumors, exhibited a much lower proliferation rate than untreated cells, suggesting that EA inhibited the cellular proliferation of ULMC. TGF-β can achieve a similar effect in place of Thunb/EA in combination [46, 47]. It should be pointed out that, since these agents can induce TGF-β production in target cells, they are suitable for cancer prevention but should be careful in administrating these agents for the purpose of treatment of established cancers.

Herbal products that inhibit TGF-β signaling in target cells

The best examples of this class of herbal products are flavenoids, such as genistein, with their ability to inhibit tumor progression and metastasis [48]. Aside from their ability to possess the anti-oxidant property, they can inhibit TGF-β signaling in the target cells, thus help to inhibit tumor growth and metastasis. The best example of this class of herbal products has been the recent paper by Lin e t al [49] who have delineated how Andrographolide down-regulates hypoxia-inducible factor-1α (HIF1α) by inhibiting TGF-β signaling in human non-small cell lung cancer A549 cells. Other natural products belonging to this class include green tea and black tea extracts [50, 51], angelica sinensis [51], machilus thunbergii [53], Chunggan extract (CGX) [54], esculentoside A [55], rhubarb extract [56], compound Astragalus and Salvia miltiorrhiza extract [57], Momordica charantia leaf extract [58], and Polypodium leucotomos [59, 60].

Herbal products that have no impact on TGF-β signaling in target cells

Herbal products such as water extracts of many Korean medicine for uterine leiomyoma and citrus unshiu (Satsuma mandarian) have no effect on TGF-β production in cancer cells under culture conditions [61, 62]. Further, lycopene is an effective preventive agent for prostate cancer but has not effect on TGF-β production in cancer cells [63-65].

Dietary factors and DNA methylation:

Diet and environmental factors directly influence epigenetic mechanisms in humans [66]. Epigenetic changes are characteristic of nearly all malignancies and include changes in DNA

methylation, histone modification and microRNAs. DNA methylation plays a critical role in cancer development and progression [66]. Alteration of DNA methylation patterns leads to deregulation of gene expression, in the absence of mutation. All tumors that have been examined show changes in DNA methylation, suggesting that this may represent a basic element of cancer biology, which bears a significant impact on tumor pathology [67-69]. There is a close relationship between the status of DNA hypermethylation and TGF-β signaling in cancer cells. TGF-β is a key regulator for DNA methylation through an increase in DNMTs expression, especially in cancer [70, 71]. There exists a differential effect of TGF-β mediated DNMT activities between benign and malignant cells. In benign cells, TGF-β inhibits DNMT expression [31, 72]. In cancer cells, TGF-β stimulates DNMT expression [19, 70]. It should be noted that, in light of the importance of both TGF-β signaling and DNA methylation in tumor progression, the majority of the methylated gene in cancer are relevant to TGF-β signaling [13]. This is consistent with our observation that over-expression of TGF-β and/or DNMTs is associated with aggressiveness and poor prognosis in prostate cancer [73, 74].

Diet and environmental factors directly influence epigenetic mechanisms in humans [66]. Dietary polyphenols from green tea, turmeric, soybeans, broccoli and others have shown to possess multiple cell-regulatory activities within cancer cells [74]. Because epigenetic deregulation occurs early in carcinogenesis and is potentially reversible, intervention strategies targeting the epigenome have been proposed for cancer prevention. Dietary components with anticancer potential, including folate, polyphenols, selenium, retinoids, fatty acids, isothiocyanates and allyl compounds, influence DNA methylation and histone modification processes [66, 74]. Such activities have been shown to affect the expression of genes involved in cell proliferation, death and differentiation that are frequently altered in cancer. **Table 2** lists selected natural products that reversed DNA hypermethylation and restore the expression of many tumor suppressor genes in target cells. Many natural products are able to reverse DNA hypermethylation through an inhibition of DNA methylatransferases (DNMT) [66]. However, the literature shows conflicting information in that some of the natural products listed in **Table 1,** especially those belonging to the class I category, resulted in an increase in DNA hypermethylation [75, 76]. This is consistent with the action of TGF-β, especially in benign cells or in early stages of cancer TGF-β inhibits DNMT expression [31, 72]. But, in advanced cancers, TGF-β stimulates the expression of DNMT [19, 70]. Therefore, natural products listed in the class I category can serve as chemo-preventive agents for cancer development, not for cancer treatment. It remains unclear, if in advanced cancer cells, these natural products are still able to inhibit DNMT expression in the presence of an aberrant signaling events of TGF-β mediated vicious cycle in tumor progression [18, 19].

(Please place **Table 1** and **Table 2** here)

Conclusion:

A variety of herbal medicine has been used either as a major medication or as a supplement either for cancer prevention or for cancer treatment. These herbal products have been administered to the population without consideration if the product is used for prevention or for treatment. As we understand the role of TGF-β in cancer cells is different from that in benign cells, we should exercise caution when we are taking these herbal products. Aside from the many effects of these herbal products on the target cells, from the point of view of TGF-β signaling, it is important that we distinguish when we are using these products for prevention or for treatment of cancer.

In light of the importance of the role of TGF-β in cancer development and in cancer progression, it is apparent that our knowledge regarding herbal products in relation to TGF-β

signaling, especially in the area of mechanism of action of each herbal compound in stimulation or inhibition of TGF-β signaling events, remains limited and justifies further investigation. It is anticipated that a significant effort can be devoted toward the investigation of relationship of herbal products in TGF-β signaling as a future direction of research in herbal medicine.

REFERENCES:

1. Anzano, M. A.; Roberts, A. B.; Meyers, C. A.; Komoriya, A.; Lamb, L. C.; *et al*. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. **1982**; 42(11):4776-8.
2. Anzano, M. A.; Roberts, A. B.; Smith, J. M.; Sporn, M. B.; De Larco, J. E. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci U S A. **1983**; 80(20):6264-8.
3. Roberts, A. B.; Frolik, C. A.; Anzano, M. A.; Sporn, M. B. Transforming growth factors from neoplastic and nonneoplastic tissues. Fed Proc. **1983**; 42(9):2621-6.
4. Massagué, J. How Cells read TGF-beta signals. Nature Rev Mol Cell Biol **2000;** 1: 169-178.
5. Derynck, R.; Akhurst, R.J.; Balmain, A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet **2001;** 29:117-129.
6. Massague, J. Cheifetz, S.; Laiho, M.; Ralph, D. A.; Weis, F.; Zentella, A. TGF-β. Cancer Surv **1992;** 12:81-103.
7. Dernyck, R.; Feng, X.; TGF-β receptor signaling. Biochem et Biophs Acta **1997,** 333:F105-F150.
8. Mu, Y.; Gudey, S. K.; Landström, M. Non-Smad signaling pathways. Cell Tissue Res. **2012;** 347(1):11-20.
9. Kang JS, Liu C, Derynck R. New regulatory mechanisms of TGF-beta receptor function. Trends Cell Biol. **2009**; 19(8):385-94.
10. Hong M, Wilkes MC, Penheiter SG, Gupta SK, *et al.* Non-Smad transforming growth factor-β signaling regulated by focal adhesion kinase binding the p85 subunit of phosphatidylinositol 3-kinase. J Biol Chem. **2011**; 286(20):17841-50.
11. Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. Proc Jpn Acad Ser B Phys Biol Sci. 2009; 85(8):314-23.
12. Lu, L.; Wang, J.; Zhang, F.; Chai, Y.; Brand, D.; *et al*. Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells. J Immunol. **2010**; 184(8):4295-306.
13. Aomatsu K, Arao T, Sugioka K, Matsumoto K, Tamura D, *et al*. TGF-β induces sustained upregulation of SNAI1 and SNAI2 through Smad and non-Smad pathways in a human corneal epithelial cell line. Invest Ophthalmol Vis Sci. **2011**; 52(5):2437-43.
14. Lamouille S, Derynck R. Emergence of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin axis in transforming growth factor-β-induced epithelial-mesenchymal transition. Cells Tissues Organs. **2011**; 193(1-2):8-22.
15. Wendt MK, Tian M, Schiemann WP. Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression. Cell Tissue Res. **2012**; 347(1):85-101.
16. Kretzschmar, M. Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/SMAD signaling defects and cancer. Breast Cancer Res. **2000;** 2(2):107-15.
17. Teicher, B. A. Malignant cells, directors of the malignant process: role of transforming growth factor-beta. Cancer Metastasis Rev. **2001;** 20(1-2):133-43.
18. Yu, N, Kozlowski, J. M.; Park, I. I.; Chen, L.; Zhang, Q.; Xu, D.; Doll, J. A.; Crawford, S. E.; Brendler, C. B.; Lee, C. Over-expression of transforming growth factor β1 in malignant prostate cells is partly caused by a runaway of TGF-β1 auto-induction mediated through a defective recruitment of protein phosphatase 2A by TGF-β type I receptor. Urology **2010;** 76(6):1519.e8-13.

19. Zhang, Q.; Chen, L.; Helfand, B.; T.; Zhu, L. J.; Kozlowski, J.; Minn, A.; Jang, T.; Yang, X. J.; Javonovic, B.; Guo, Y.; Lonning, S.; Harper, J.; Teicher, B. A.; Yu, N.; Brendler, C.; Wang, J.; Catalona, W. J.; Lee, C. Transforming Growth Factor-beta-induced DNA methyltransferase contributes to aggressive prostate cancer phenotypes and predicts biochemical recurrence after radical prostatectomy PloS ONE **2011;** 6:e25168.
20. Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, et al. Transactivation of Vimentin by β-Catenin in Human Breast Cancer Cells. Cancer Res **2003**; 63:2658-64.
21. Serra R, Easter SL, Jiang W, Baxley SE. Wnt5a as an effector of TGFβ in mammary development and cancer. J Mammary Gland Biol Neoplasia. **2011**; 16(2):157-67.
22. Shibata, S.; Marushima, H.; Asakura, T.; Matsuura, T.; Eda, H.; Aoki, K.; Matsudaira, H.; Ueda, K.; Ohkawa, K. Three-dimensional culture using a radial flow bioreactor induces matrix metalloprotease 7-mediated EMT-like process in tumor cells via TGFbeta1/Smad pathway. Int J Oncol. **2009**; 34(5):1433-48.
23. Briones-Orta MA, Tecalco-Cruz AC, Sosa-Garrocho M, Caligaris C, Macías-Silva M. Inhibitory Smad7: emerging roles in health and disease. Curr Mol Pharmacol. **2011**; 4(2):141-53.
24. Weidenaar AC, ter Elst A, Kampen KR, Meeuwsen-de Boer TG, de Jonge HJ, Scherpen FJ, et al. Stromal interaction essential for vascular endothelial growth factor A-induced tumour growth via transforming growth factor-β signalling. Br J Cancer **2011**; 105:1856-63.
25. Fukukawa C, Tanuma N, Okada T, Kikuchi K, Shima H. pp32/ I-1(PP2A) negatively regulates the Raf-1/MEK/ERK pathway. Cancer Lett. **2005**; 226(2):155-60.
26. Letourneux, C.; Rocher, G.; Porteu, F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. EMBO J **2006**; 25:727-38.
27. Kim, S. I.; Kwak, J. H.; Wang, L.; Choi, M. E. Protein phosphatase 2A is a negative regulator of transforming growth factor-beta1-induced TAK1 activation in mesangial cells. J Biol Chem **2008**; 283:10753-63.
28. Jiang, Y. G.; Luo, Y.; He, D. L.; Li, X.; Zhang, L. L.; Peng, T.; et al. Role of Wnt/β –catenin signaling in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1α. Int J of Urol **2007**; 14:1034-9.
29. Zhang, Q.; Yang, X.; Pins, M.; Javonovic, B.; Kuzel, T.; Kim, S. J. et al. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. Cancer Res **2005**; 65:1761–9.
30. Petersen M, Pardali E, van der Horst G, Cheung H, van den Hoogen C, van der Pluijm G, et al. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene **2010;**29:1351-61.
31. Luo, X.; Zhang, Q.; Liu, V.; Xia, Z.; Pothoven, K. L.; Lee, C. Cutting Edge: TGF-β Induced expression of Foxp3 in T cells is mediated through inactivation of ERK. Journal of Immunology **2008;** 180:2757-2761.
32. Bhowmick, N. A,; Chytil, A.; Plieth, D.; Gorska, A. E.; Dumont, N.; *et al*. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science. **200**4; 303(5659):848-51.
33. Ao, M.; Franco, O. E.; Park, D.; Raman, D.; Williams, K.; *et al*. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. Cancer Res. **2007**; 67(9):4244-53.
34. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer. 2008; 123(10):2229-38.

35. Rowley DR. What might a stromal response mean to prostate cancer progression? Cancer Metastasis Rev. **1998**; 17(4):411-9.
36. Verrecchia F, Chu ML, Mauviel A. Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J Biol Chem. **2001**; 276(20):17058-62.
37. Li, X.; Sterling, J. A.; Fan, K. H.; Vessella, R. L.; Shyr. Y.; *et al*. Loss of TGF-β Responsiveness in Prostate Stromal Cells Alters Chemokine Levels and Facilitates the Development of Mixed Osteoblastic/Osteolytic Bone Lesions. Mol Cancer Res. **2012**; (In Press)
38. Hodkinson PS, Mackinnon AC, Sethi T. Extracellular matrix regulation of drug resistance in small-cell lung cancer. Int J Radiat Biol. **2007**; 83(11-12):733-41.
39. Sakko AJ, Ricciardelli C, Mayne K, Suwiwat S, LeBaron RG, *et al*. Modulation of prostate cancer cell attachment to matrix by versican. Cancer Res. **2003;** 63(16):4786-91.
40. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, *et al*. Tumor induction of VEGF promoter activity in stromal cells. Cell. **1998**; 94(6):715-25.
41. Funahashi, H.; Imai, T.; Tanaka, Y.; Tsukamura, K.; Hayakawa, Y.; Kikumori, T.; Mase, T.; Itoh, T.; Nishikawa, M.; Hayashi, H.; Shibata, A.; Hibi, Y.; Takahashi, M.; Narita, T. Wakame seaweed suppresses the proliferation of 7,12-dimethylbenz(a)-anthracene-induced mammary tumors in rats. Jpn J Cancer Res. **1999;** 90(9):922-7.
42. Lu, R.; Serrero, G.; Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol. **1999,**179(3):297-304.
43. Yamamoto, M.; Ogawa, K.; Morita, M.; Fukuda, K.; Komatsu, Y. The herbal medicine Inchin-ko-to inhibits liver cell apoptosis induced by transforming growth factor beta 1. Hepatology. **1996;** 23(3):552-9.
44. Chou, C. C.; Pan, S. L.; Teng, C. M.; Guh, J. H. Pharmacological evaluation of several major ingredients of Chinese herbal medicines in human hepatoma Hep3B cells. Eur J Pharm Sci. **2003;** 19(5):403-12.
45. Lee, C. Y.; Hsu, Y. C.; Wang, J. Y.; Chen, C. C.; Chiu, J. H. Chemopreventive effect of selenium and Chinese medicinal herbs on N-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma in Syrian hamsters. Liver Int. **2008;** 28(6):841-55.
46. Lee, T. K.; Kim, D. I.; Han, J. Y.; Kim, C. H. Inhibitory effects of Scutellaria barbata D. Don. and Euonymus alatus Sieb. on aromatase activity of human leiomyomal cells. Immunopharmacol Immunotoxicol. **2004;** 26(3):315-27.
47. Lee, T. K.; Lee, J. Y.; Kim, D. I.; Lee, Y. C.; Kim, C.H. Differential regulation of protein kinase C activity by modulating factors and Euonymus alatus (Thunb.) Sieb in human myometrial and uterine leiomyomal smooth muscle cells. Int J Gynecol Cancer. **2005;** 15(2):349-58.
48. McCarty, M. F. Isoflavones made simple - genistein's agonist activity for the beta-type estrogen receptor mediates their health benefits. Med Hypotheses. **2006;** 66(6):1093-114.
49. Lin, H. H.; Tsai, C. W.; Chou, F. P.; Wang, C. J.; Hsuan, S. W.; Wang, C. K.; Chen, J. H.; Andrographolide down-regulates hypoxia-inducible factor-1α in human non-small cell lung cancer A549 cells. Toxicol Appl Pharmacol. **2011;** 250(3):336-45.
50. Roomi, M. W.; Roomi, N.; Ivanov, V.; Kalinovsky, T.; Niedzwiecki, A.; Rath, M. Inhibitory effect of a mixture containing ascorbic acid, lysine, proline and green tea extract on critical parameters in angiogenesis. Oncol Rep. **2005;** 14(4):807-15.
51. Mandal, D.; Bhattacharyya, S.; Lahiry, L.; Chattopadhyay, S.; Sa, G.; Das, T. Black tea-induced decrease in IL-10 and TGF-beta of tumor cells promotes Th1/Tc1 response in tumor bearer. Nutr Cancer. **2007;** 58(2):213-21.

52. Han, G.; Zhou, Y. F.; Zhang, M. S.; Cao, Z.; Xie, C. H.; Zhou, F. X.; Peng, M.; Zhang, W. J. Angelica sinensis down-regulates hydroxyproline and Tgfb1 and provides protection in mice with radiation-induced pulmonary fibrosis. Radiat Res. **2006;** 165(5):546-52.
53. Park, E. Y.; Shin, S. M.; Ma, C. J.; Kim, Y. C.; Kim, S. G. Meso-dihydroguaiaretic acid from Machilus thunbergii down-regulates TGF-beta1 gene expression in activated hepatic stellate cells via inhibition of AP-1 activity. Planta Med. **2005;** 71(5):393-8.
54. Shin, J. W.; Son, J. Y.; Oh, S. M.; Han, S. H.; Wang, J. H.; Cho, J. H.; Cho, C. K.; Yoo, H. S.; Lee, Y. W.; Lee, M. M.; Hu, X. P.; Son, C. G. An herbal formula, CGX, exerts hepatotherapeutic effects on dimethylnitrosamine-induced chronic liver injury model in rats. World J Gastroenterol. **2006;** 12(38):6142-8.
55. Xiao, Z.; Su, Y.; Yang, S.; Yin, L.; Wang, W .; Yi, Y, Fenton BM, Zhang L, Okunieff P. Protective effect of esculentoside A on radiation-induced dermatitis and fibrosis. Int J Radiat Oncol Biol Phys. **2006;** 65(3):882-9.
56. Yu, H. M.; Liu, Y. F.; Cheng, Y. F.; Hu, L. K.; Hou, M. Effects of rhubarb extract on radiation induced lung toxicity via decreasing transforming growth factor-beta-1 and interleukin-6 in lung cancer patients treated with radiotherapy. Lung Cancer. **2008;** 59(2):219-26.
57. Liu, X.; Yang, Y.; Zhang, X.; Xu, S.; He, S.; Huang, W.; Roberts, M. S. Compound Astragalus and Salvia miltiorrhiza extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell. J Gastroenterol Hepatol. **2010;** 25(2):420-6.
58. Pitchakarn, P.; Ogawa, K.; Suzuki, S.; Takahashi, S.; Asamoto, M.; Chewonarin, T.; Limtrakul, P.; Shirai, T. Momordica charantia leaf extract suppresses rat prostate cancer progression in vitro and in vivo. Cancer Sci. **2010,** 101(10):2234-40.
59. Philips, N.; Dulaj, L.; Upadhya, T. Cancer cell growth and extracellular matrix remodeling mechanism of ascorbate; beneficial modulation by P. leucotomos. Anticancer Res. **2009;** 29(8):3233-8.
60. Philips, N.; Conte, J.; Chen, Y. J.; Natrajan, P.; Taw, M.; Keller, T.; Givant, J.; Tuason, M.; Dulaj, L.; Leonardi, D.; Gonzalez, S. Beneficial regulation of matrixmetalloproteinases and their inhibitors, fibrillar collagens and transforming growth factor-beta by Polypodium leucotomos, directly or in dermal fibroblasts, ultraviolet radiated fibroblasts, and melanoma cells. Arch Dermatol Res. **2009,** 301(7):487-95.
61. Bajracharya, P.; Lee, E. J.; Lee, D. M.; Shim, S. H.; Kim, K. J.; Lee, S. H.; Bae, J. J.; Chun, S. S.; Lee, T. K.; Kwon, S. H.; Choi, I. Effect of different ingredients in traditional Korean medicine for human uterine leiomyoma on normal myometrial and leiomyomal smooth muscle cell proliferation. Arch Pharm Res. **2009;** 32(11):1555-63.
62. Lee, S.; Ra, J.; Song, J. Y.; Gwak, C.; Kwon, H. J.; Yim, S. V.; Hong, S. P.; Kim, J. I.; Lee, K. H.; Cho, J. J.; Park, Y. S.; Park, C. S.; Ahn, H. J. Extracts from Citrus unshiu promote immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model. J Ethnopharmacol. **2011;** 133(3):973-9.
63. Gunasekera, R. S.; Sewgobind, K.; Desai, S.; Dunn, L.; Black, H. S.; McKeehan, W. L.; Patil, B. Lycopene and lutein inhibit proliferation in rat prostate carcinoma cells. Nutr Cancer. **2007;** 58(2):171-7.
64. Wertz, K.; Siler, U.; Goralczyk, R. Lycopene: modes of action to promote prostate health. Arch Biochem Biophys. **2004;** 430(1):127-34.
65. Nantz, M. P.; Rowe, C. A.; Nieves, C. Jr.; Percival, S. S. Immunity and antioxidant capacity in humans is enhanced by consumption of a dried, encapsulated fruit and vegetable juice concentrate. J Nutr. **2006;** 136(10):2606-10.

66. Link, A.; Balaguer, F.; Goel, A. Cancer chemoprevention by dietary polyphenols: promising role for epigenetics. Biochem Pharmacol. **2010,** 80(12):1771-92.
67. Cedar H, Bergman Y. Programming of DNA Methylation Patterns. Annu Rev Biochem. **2012;** (In Press)
68. Chiam K, Ricciardelli C, Bianco-Miotto T. Epigenetic Biomarkers in Prostate Cancer: Current and Future Uses. Cancer Lett. **2012;** (In Press).
69. Sandoval, J.; Esteller, M. Cancer epigenomics: beyond genomics. Curr Opin Genet Dev. **2012;** 22(1):50-5
70. Matsumura, N.; Huang, Z.; Mori, S.; Baba, T.; Fujii, S.; Konishi, I.; Iversen, E. S.; Berchuck, A.; Murphy, S. K. Epigenetic suppression of the TGF-beta pathway revealed by transcriptome profiling in ovarian cancer. Genome Res. **2011;** 21(1):74-82.
71. Zhang, D.; Al-Hendy, M.; Richard-Davis, G.; Montgomery-Rice, V.; Rajaratnam, V.; Al-Hendy, A. Antiproliferative and proapoptotic effects of epigallocatechin gallate on human leiomyoma cells. Fertil Steril. **2010;** 94(5):1887-93.
72. You, H.; Ding, W.; Rountree, C. B. Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. Hepatology. **2010;** 51(5):1635-44.
73. Zhang, Q.; Helfand, B. T.; Jang, T. L.; Zhu, L. J.; Chen, L.; Yang, X. J.; Kozlowski, J.; Smith, N.; Kundu, S. D.; Yang, G.; Raji, A. A.; Javonovic, B.; Pins, M.; Lindholm, P.; Guo, Y.; Catalona, W. J.; Lee, C. NF-kB-Mediated Transforming Growth Factor-β-Induced Expression of Vimentin is an Independent Predictor of Biochemical Recurrence After Radical Prostatectomy. Clinical Cancer Res **2009;** 15:3557-3567.
74. Lim, U.; Song, M. A. Dietary and lifestyle factors of DNA methylation. Chapter 23. Methods Mol Biol **2012**; 863:359-376.
75. Chuang, J. C.; Yoo, C. B.; Kwan, J. M.; Li, T. W.; Liang, G.; Yang, A. S.; Jones, P. A. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. Mol Cancer Ther. **2005;** 4(10):1515-20.
76. Stresemann, C.; Brueckner, B.; Musch, T.; Stopper, H.; Lyko, F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res. **2006;** 66(5):2794-800.
77. Jang, M.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol. Drugs Exp Clin Res. **1999,** 25(2-3):65-77.
78. Whyte, L.; Huang, Y. Y.; Torres, K.; Mehta, R. G. Molecular mechanisms of resveratrol action in lung cancer cells using dual protein and microarray analyses. Cancer Res. **2007;** 67(24):12007-17.
79. Tamura, T.; Kobayashi, H.; Yamataka, A.; Lane, G. J.; Koga, H.; Miyano, T. Inchin-ko-to prevents medium-term liver fibrosis in postoperative biliary atresia patients. Pediatr Surg Int. **2007;** 23(4):343-7.
80. Kitano, A.; Saika, S.; Yamanaka, O.; Reinach, P. S.; Ikeda, K.; Okada, Y.; Shirai, K.; Ohnishi, Y. Genipin suppression of fibrogenic behaviors of the alpha-TN4 lens epithelial cell line. J Cataract Refract Surg. **2006**; 32(10):1727-35.
81. Vittal, R.; Selvanayagam, Z. E.; Sun, Y.; Hong, J.; Liu, F.; Chin, K. V.; Yang, C. S. Gene expression changes induced by green tea polyphenol (-)-epigallocatechin-3-gallate in human bronchial epithelial 21BES cells analyzed by DNA microarray. Mol Cancer Ther. **2004;** 3(9):1091-9.
82. Manabe, M.; Takenaka, R.; Nakasa, T.; Okinaka, O. Induction of anti-inflammatory responses by dietary Momordica charantia L. (bitter gourd). Biosci Biotechnol Biochem. **2003;** 67(12):2512-7.
83. Ruiz-Torres, M. P.; Perez-Rivero, G.; Diez-Marques, M. L.; Griera, M.; Ortega, R.; Rodriguez-Puyol, M.; Rodríguez-Puyol, D. Role of activator protein-1 on the effect of arginine-glycine-aspartic

acid containing peptides on transforming growth factor-beta1 promoter activity. Int J Biochem Cell Biol. **2007;** 39(1):133-45.
84. Pavese, J. M.; Farmer, R. L.; Bergan, R. C. Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Rev. **2010**; 29(3):465-82.
85. Ji, G,; Yang, Q.; Hao, J.; Guo, L.; Chen, X.; Hu, J.; Leng, L.; Jiang, Z. Anti-inflammatory effect of genistein on non-alcoholic steatohepatitis rats induced by high fat diet and its potential mechanisms. Int Immunopharmacol. **2011**; 11(6):762-8.
86. Kwak, K. G.; Wang, J. H.; Shin, J. W.; Lee, D. S.; Son, C. G. A traditional formula, Chunggan extract, attenuates thioacetamide-induced hepatofibrosis via GSH system in rats. Hum Exp Toxicol. **2011**; 30(9):1322-32.
87. Li, G. S.; Jiang, W. L.; Tian, J. W.; Qu, G. W.; Zhu, H. B.; Fu, F. H. In vitro and in vivo antifibrotic effects of rosmarinic acid on experimental liver fibrosis. Phytomedicine. **2010**; 17(3-4):282-8.
88. Adjakly, M.; Bosviel, R.; Rabiau, N.; Boiteux, J. P.; Bignon, Y. J.; Guy, L.; Bernard-Gallon, D. DNA methylation and soy phytoestrogens: quantitative study in DU-145 and PC-3 human prostate cancer cell lines. Epigenomics. **2011,** 3(6):795-803.
89. King-Batoon, A.; Leszczynska, J. M.; Klein, C. B. Modulation of gene methylation by genistein or lycopene in breast cancer cells. Environ Mol Mutagen. **2008;** 49(1):36-45.
90. Liu, A.G.; Erdman, J. W. Jr. Lycopene and apo-10'-lycopenal do not alter DNA methylation of GSTP1 in LNCaP cells. Biochem Biophys Res Commun. **2011;** 412(3):479-82.
91. Khor, T. O.; Huang, Y.; Wu, T. Y.; Shu, L.; Lee, J.; Kong, A. N. Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation. Biochem Pharmacol. **2011;** 82(9):1073-8.
92. Liu, Y. L.; Yang, H. P.; Gong, L.; Tang, C. L.; Wang, H. J. Hypomethylation effects of curcumin, demethoxycurcumin and bisdemethoxycurcumin on WIF-1 promoter in non-small cell lung cancer cell lines. Mol Med Report. **2011;** 4(4):675-9.
93. Papoutsis, A. J. ; Borg, J. L. ; Selmin, O. I.; Romagnolo, D. F. BRCA-1 promoter hypermethylation and silencing induced by the aromatic hydrocarbon receptor-ligand TCDD are prevented by resveratrol in MCF-7 Cells. J Nutr Biochem. **2011;** (In Press)
94. Zhu, W.; Qin, W.; Zhang, K.; Rottinghaus, G. E.; Chen, Y. C.; Kliethermes, B.; Sauter, E. R. Trans-Resveratrol Alters Mammary Promoter Hypermethylation in Women at Increased Risk for Breast Cancer. Nutr Cancer. **2012;** (In Press)
95. Paluszczak, J.; Krajka-Kuźniak, V.; Baer-Dubowska, W. The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells. Toxicol Lett. **2010**; 192(2):119-25.
96. Majid, S.; Dar, A. A.; Shahryari, V.; Hirata, H.; Ahmad, A.; Saini, S.; Tanaka, Y.; Dahiya, A. V.; Dahiya, R. Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. Cancer. **2010;** 116(1):66-76.
97. Fang, M,; Chen, D.; Yang, C. S. Dietary polyphenols may affect DNA methylation. J Nutr. **2007;** 137(1 Suppl):223S-228S.
98. Li, Y.; Tollefsbol, T. O. Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. Curr Med Chem. **2010;** 17(20):2141-51.

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Table 1: Three classes of natural products related to the effect on TGF-β signaling:

Name	Description	References
Class I: natural products that induce TGF-β production in target cells*		
Seaweed	A natural source of iodine	[41]
Resveratrol	A natural phytoestrogen in red wine	[42, 77, 78]
Inchin-ko-to (ICKT)	An ancient oriental herbal formulation for jaundice	[79]
Genipin	A metabolite component of inchin-ko-to	[80]
Scutellaria baicalensis Georgi	A herbal medicine for liver diseases	[45]
Gui-jun woo	A Korean herbal medicine used for treatment of tumors	[46, 47]
EGCG	A natural product of tea extracts	[71, 81]
Momordica charantia	Leaf extract from bitter melon	[56, 82]
Polypodium leucotomos	A tropical fern plant	[59, 60]
Curcumin	Active components of spice turmeric	[83]
Class II: natural products that inhibit TGF-β signaling**		
Genistein	An active flavonoid in soy	[84, 85]
Long Dan Tan	A herbal medicines for chronic liver disease	[44]
Andrograpgolide	A diterpenoid lactone from a traditional herbal medicine	[49]
Angelica Sinensis	The root of *Angelica sinensis*, known as *Danggui*	[52]
Machilus	Barks of *Machilus* DGA is glycosidic triterpene alkaloids	[53]
Chunggan extract	A hepatotherapeutic herbal formula	[54, 86]
Esculentoside A	A saponin isolated from herb *phytolacca esculenta*,	[55]
Rhubarb (*dahuang*)	Extractof the dried radix and rhizome of *Rheum palmatum* L	[56]
Compound Astragalus Salvia miltiorrhiza extract (CASE)	Extract of Leguminosae and Lamiaceae	[57]
Polypodium leucotomos	A tropical fern plant	[59, 60]
Rosmarinic acid	Naturally occurring polyphenol in Labitae plants	[87]
Class III: natural products that have no impact on TGF-β production***		
Lycopene	A natural product of tomato	[63-65]
Satsuma mandarian	The peel of citrus fruit	[61, 62]

***Since these agents can induce TGF-β production in target cells, they are suitable for cancer prevention. But, we should exercise caution, when using these agents for the purpose of established cancers**.

****These agents listed in Class II are known to inhibit TGF-β signaling. Aside from their anti-oxidant property, they can be used to help to inhibit tumor growth and metastasis.**

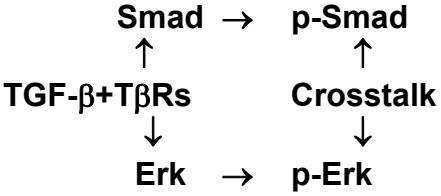
*****Although these products have established anti-cancer effects, they have nothing to do with TGF-β signaling. Therefore, no recommendation will be offered regarding their administration**

Table 2: Natural products that are able to reverse DNA hypermethylation in target cells

Name	Description	References
EGCG	A natural product of tea extracts	[97, 98]
Genistein	An active flavonoid in soy	[88, 89, 96]
Lycopene	A natural product of tomato	[89, 90]
Curcumin	Active components of the spice turmeric	[91, 92]
Resveratrol	A natural phytoestrogen in red wine	[93, 94]
Rosmarinic acid	Naturally occurring polyphenol in Labitae plants	[95]

This list does not intend to be comprehensive. Rather, these products all have a common property of inhibiting DNMT expression.

Figure 1: Biology of TGF-β signaling.



The biological effect of TGF-β is mediated through type I, type II receptors. Upon engagement with TGF-β receptors (TβRs), the complex activates both Smad2/3 and/or Erk1/2 through phosphorylation. Phosphorylation of Smad2/3 is, the classical pathway for TGF-β signaling. The non-Smad pathways (or the non-classical pathway) of TGF-β, have also been identified, which activates (or phosphorylates Erk, PI3K/AKT, PP2A/p70s6K, and JNK. In general, events mediated through the Smad pathways are mainly related to growth arrest and apoptosis; while those mediated through the non-Smad pathways, especially through Erk phosphorylation, are mainly related to cell proliferation and migration. In benign cells, there exists a crosstalk between the classical and non-classical pathways of TGF-β signaling to maintain the structural and functional integrity and tissue homeostasis. In cancer cells, especially in advanced cancer, the non-classical TGF-β signaling is the predominant pathway; while the Smad mediated pathway is limited. The Erk dominated signaling pathway in cancer cells creates a unique microenvironment leading to a vicious cycle of a continuous tumor growth and progression.